PHARMEUROPA BIO & SCIENTIFIC NOTES

Scientific articles on pharmacopoeial matters

EDQM
December 2018

edqm
European Directorate for the Quality of Medicines & HealthCare | Direction européenne de la qualité du médicament & soins de santé

COUNCIL OF EUROPE
CONSEIL DE L'EUROPE

CONTRIBUTIONS

The Editor is pleased to consider contributions for publication in *Pharmeuropa Bio & Scientific Notes* provided they have some relationship with the work of the European Pharmacopoeia. Please consult the Instructions for authors before submitting articles to the Secretariat.

The EDQM recalls that authors alone are responsible for the views expressed in signed contributions.

Articles published in *Pharmeuropa Bio & Scientific Notes* are indexed in the PubMed database of the National Library of Medicine.

COPYRIGHT

ISSN: 2075-2504

European Directorate for the Quality of Medicines & HealthCare (EDQM)
Council of Europe – 7 allée Kastner
CS 30026, F-67081 STRASBOURG, FRANCE

Tel: +33 (0)3 88 41 30 30

Internet: www.edqm.eu
FAQ & EDQM HelpDesk: www.edqm.eu/hd

CONTENTS

Establishment of detection antibodies BRRs batch 4 for *in vitro* potency assay of hepatitis A vaccines by ELISA

S. Morgeaux[1], A. Koy[1], I. Manniam[1], E. Regourd[2], P. Variot[1], A. Costanzo[2]

ABSTRACT

The European Pharmacopoeia (Ph. Eur.) standard ELISA method for determination of antigen content of hepatitis A vaccines (HAV) requires specific coating and detection Biological Reference Reagents (BRRs). The 3rd batch of detection antibodies BRRs was established in 2015 for use in conjunction with the Ph. Eur. general chapter 2.7.14 'Assay of hepatitis A vaccine'. Stocks of these BRRs were running low and therefore the European Directorate for the Quality of Medicines & HealthCare (EDQM) organised a collaborative study to qualify replacement batches. The candidate BRR antibodies batch 4 were prepared under appropriate conditions from starting materials similar to previous batches to ensure continuity. During the collaborative study, the new batches of antibodies were compared to previous batches of BRRs. Results confirmed that they were suitable to be used for the intended purpose, and could be used at the same final concentrations as the previous batch, i.e. 1:500 for the primary antibody and 1:400 for the conjugated secondary antibody. They were adopted in June 2017 by the Ph. Eur. Commission as Hepatitis A virus primary detection antibody BRR batch 4 and Conjugated secondary detection antibody BRR batch 4, respectively. They are available from the EDQM as Hepatitis A vaccine ELISA detection antibodies set BRR batch 4.

KEYWORDS

Hepatitis A vaccine, HAV, ELISA, collaborative study, European Pharmacopoeia, Biological Reference Reagents.

1. INTRODUCTION

The standard ELISA method for the determination of antigen content in adsorbed hepatitis A vaccines (HAV) was established in 2012 during the course of a collaborative study (BSP107) organised in the framework of the Biological Standardisation Programme (BSP) by the European Directorate for the Quality of Medicines & HealthCare (EDQM) [1]. This method, described in the European Pharmacopoeia (Ph. Eur.) general chapter 2.7.14 *Assay of hepatitis A vaccine* [2], requires reference reagents i.e. a coating reagent and detection antibodies (anti-hepatitis A virus primary detection antibody (MAbs) and Horseradish peroxidase (HRPO)-conjugated secondary detection antibody (HRPO-GAM)). The first 2 batches of these reagents were calibrated during the same study. They were subsequently adopted as Biological Reference Reagents (BRRs) by the Ph. Eur. Commission in November 2012. The subsequent batch of detection antibodies (batch 3) was established in 2015 [3] and its stocks were running

1 S. Morgeaux. A. Koy, I. Manniam, P. Variot, Agence Nationale de Sécurité du Médicament et des Produits de Santé (ANSM), Pôle libération de lots et surveillance du marché des produits biologiques, Direction des Contrôles, Lyon, France.

2 E. Regourd, A. Costanzo (corresponding author's e-mail: angele.costanzo@edqm.eu), European Directorate for the Quality of Medicines & HealthCare, Department of Biological Standardisation, OMCL Network & HealthCare (DBO), Council of Europe, Strasbourg, France.

low. Therefore, the EDQM organised a collaborative study to qualify replacement batches. Dr S. Morgeaux (ANSM, France) was nominated as project leader by the BSP Steering Committee. The candidate BRR (cBRR) antibodies batch 4 were prepared from starting materials similar to those used to prepare batch 3, i.e. from the same manufacturers but from different production batches. Several vials of each antibody stock solution were diluted at the EDQM (see below for details), pooled and, after working dilutions had been defined for each antibody diluted stock in preliminary assays, suitable aliquots were produced at the EDQM. The study was run in several phases:

- a preliminary phase for determination of appropriate working dilutions after dilution of the stock solutions;
- after aliquoting, testing of the aliquots to verify their activity (Phase 1); and
- a collaborative study (Phase 2) for final qualification.

To ensure continuity, the candidate materials were compared to the BRRs batch 3 during Phase 1 of the project using the 1st WHO International Standard (IS) for HAV inactivated (95/500) [4] and the Ph. Eur. HAV (inactivated, non-adsorbed) BRP batch 2 (BRP2) [5] as test samples. As the outcome of the preliminary qualification phases was satisfactory, the collaborative study was started. The experimental phase ran from December 2016 to February 2017 and the results are summarised herein.

2. PARTICIPANTS

Three Official Medicines Control Laboratories (OMCLs) took part in the study. Participants are listed in section 8. For the study, they were given arbitrary code numbers not necessarily linked to the order of listing.

3. MATERIALS, METHODS AND STUDY DESIGN

Specific ELISA reagents (BRRs and cBRRs) were distributed to participants by the EDQM. Other non-specific reagents, such as ingredients for the preparation of buffers, were procured by the participants from commercial sources. The composition of buffers and the Standard Operating Procedure applied here for the ELISA can be found in the Ph. Eur. general chapter 2.7.14 *Assay of hepatitis A vaccine* [2]. A study protocol was nevertheless provided to participants to outline the specific sample dilutions and plate layouts to be applied.

3.1. Materials

3.1.1. Common ELISA reagents

Participants were instructed to handle the reagents as appropriate in order to avoid microbial contamination and to place the BRRs back to − 20 °C immediately after having taken out the volume necessary to perform the assay.

Coating reagent and detection antibodies BRR

- The stock solution of hepatitis A virus coating reagent for ELISA BRR batch 1 (catalogue number Y0001624) was to be stored at − 20 °C until use. Participants were instructed to prepare a fresh working solution just before use by diluting the stock solution 1:500 in carbonate-bicarbonate buffer 0.05 M pH 9.6 [1, 2].
- The Hepatitis A vaccine ELISA detection antibodies set BRR batch 3 (catalogue number Y0001623) is composed of the anti-hepatitis A virus primary detection antibody BRR batch 3 (mouse monoclonal antibodies (MAbs)) and of the conjugated secondary detection antibody BRR batch 3 (HRPO-labelled goat anti-mouse antibodies (HRPO-GAM)). They are herein referred to as ‘current MAbs’ and ‘current HRPO-GAM’ respectively. The stock solutions were to be stored at − 20 °C upon receipt by participants and diluted just

before use 1:500 and 1:400 respectively, in Phosphate Buffered Saline – Bovine Serum Albumin – Tween (PBS-B-T) buffer [1, 2].

Candidate detection antibodies BRRs

Vials of a new batch of anti-HAV monoclonal antibody (herein referred to as 'new MAbs') were procured and stored at + 5 °C until use. Three vials of stock solution were diluted 1:10 in PBS/glycerol (1:1, *V/V*) and all solutions obtained were then pooled. The diluted stock was stored at − 20 °C. Similarly, the vials of a new batch of conjugated secondary antibody (herein referred to as 'new HRPO-GAM') were diluted, pooled, and stored at − 20 °C.

For the preliminary tests to determine the appropriate working concentration, fresh working dilutions of each detection antibody were prepared from the diluted stock just before testing by dilution into PBS-B-T buffer. After determination of the appropriate working dilution to be applied, the diluted solutions were aliquoted at the EDQM into appropriate, pre-cooled, sterile 0.5 mL screw cap tubes. Each tube contains a volume sufficient for at least five 96-well ELISA plates. The HRPO-GAM was aliquoted in amber tubes to protect the contents from light during storage and handling.

3.1.2. Test samples

- The 1st WHO IS for HAV, inactivated (95/500) [4] was kindly provided by the National Institute for Biological Standards and Control (NIBSC). The IS has an assigned antigen content of 100 IU/mL.
- The Ph. Eur. HAV (inactivated, non-adsorbed) BRP batch 2, herein referred to as BRP2, has an assigned antigen content of 1350 IU/mL [5]. The BRP2 was thawed by participants, distributed into aliquots and re-frozen below − 50 °C immediately thereafter, as prescribed in the accompanying leaflet provided with the study protocol. One aliquot was tested in each assay.

3.2. Methods and study design

As past experiences showed that the activity of specific monoclonal antibodies used for this method is stable from batch to batch, the the candidate primary monoclonal antibody was tested at the same dilution as used for previous batches, i.e. 1:500. In contrast, the activity of the secondary HRPO-conjugated polyclonal detection antibody is more variable and known to significantly fluctuate from one vial of starting material to another, even within the same lot number. It was therefore decided to test the new HRPO-GAM at 2 different dilutions (1:400 and 1:800) in the preliminary qualification. These experiments allowed the identification of a suitable working dilution for both new antibody batches, i.e. 1:500 for the new MAbs and 1:400 for the new HRPO-GAM. These were then to be confirmed in additional laboratories during a small collaborative study in order to avoid biases.

Participants in the collaborative study were instructed to test both test samples in duplicate in 3 independent assays, preferably on different days, using the pre-defined working dilutions for the cBRRs. They were asked to report data for individual OD measurements at 405 nm, as well as the calculated estimates for the BRP2 relative to the IS. To allow comparison, the BRRs batch 3 were also included by one of the participating laboratories. As the measurement window (difference between background level and maximal optical density (ODmax)) is also an important parameter to consider in this type of assay, ODmax levels reached using the cBRRs were compared with the levels found with the BRRs batch 3 within the present study and also to those observed in predecessor studies, for the sake of continuity.

4. RESULTS

All participants reported data from 3 valid ELISA tests as requested. They submitted individual OD values and antigen content calculations for the BRP2 against the IS. The latter were recalculated at the EDQM using the same statistical method as used for the predecessor studies [1, 5]. For laboratory 1, the OD data from the 2 first dilutions of the IS were excluded in assay 3 because of a large difference in duplicates which was not observed for the other assays or for BRP2, suggesting a technical problem with these particular assay wells. For laboratory 2, the OD data from the 2 last dilutions of the BRP2 were excluded in assay 1 because a handling error was found and reported by the laboratory with regard to these wells.

Some assays showed significant deviations from linearity and/or parallelism ( ). However, the weighted correlation coefficient was at least 0.99 and therefore they could be retained as valid and were included in the overall calculation as described previously [1]. Consequently, all assays were considered statistically valid.

4.1. Concentration-response curves

Table 1 summarises the ODmax values (mean of duplicates per assay) obtained by the 3 participating laboratories for the IS and BRP2 using the cBRRs and the BRRs batch 3 (the latter in laboratory 3 only). The means of means per laboratory and corresponding standard deviation (SD) were also calculated. Levels of background signal are also indicated as means of n ≥ 20 data points per assay.

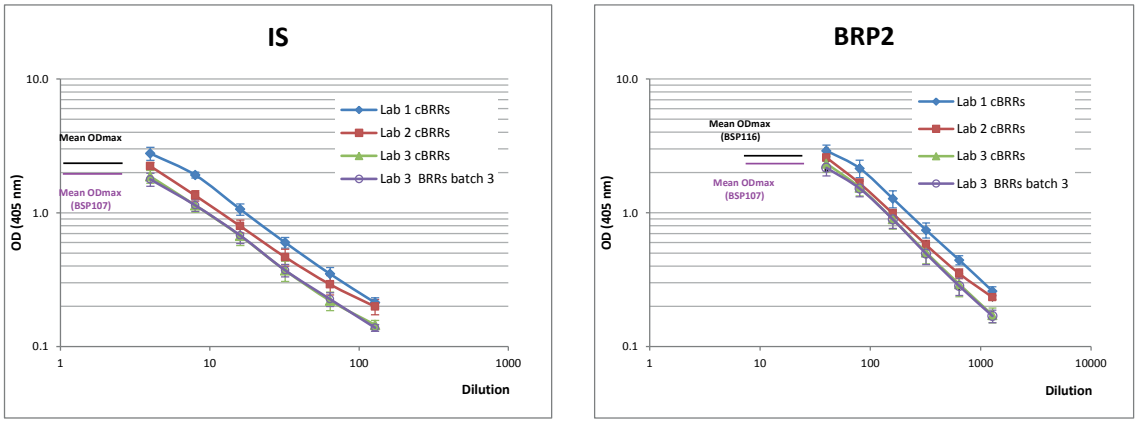
Table 1 – *Summary of ODmax values and blanks per assay and laboratory*

Lab	Assay	BRRs batch 3							cBRRs						
		IS (pre-dil. 1/4)	Mean	SD	BRP (pre-dil. 1/40)	Mean	SD	Blank	IS (pre-dil. 1/4)	Mean	SD	BRP (pre-dil. 1/40)	Mean	SD	Blank
1	1								2.525	2.776	0.35	2.996	2.912	0.32	0.085
	2								3.027			3.186			0.076
	3								inv.			2.554			0.078
2	1								2.132	2.230	0.15	2.499	2.584	0.16	0.098
	2								2.406			2.774			0.103
	3								2.151			2.479			0.092
3	1	1.996	1.778	0.22	2.442	2.168	0.30	0.071	1.928	1.868	0.21	2.350	2.336	0.34	0.071
	2	1.564			1.844			0.081	1.630			1.993			0.081
	3	1.775			2.217			0.072	2.047			2.665			0.072

Inv.: invalid. Shaded cells: not tested.

The data demonstrates satisfactory consistency of the observed ODs, in particular the ODmax levels, between assays and laboratories. Background detection (referred to as 'Blank' in) is generally low and consistent with background values observed in previous studies. Raw data of individual OD values are given in appendix for information.

Figure 1 – *Concentration – response curves (mean of 3 assays/laboratory) using the IS and BRP2 as test samples*



A graphical representation of the concentration-response profiles is given in Figure 1 (mean of 3 assays with standard deviation error bars). The ODmax levels reached for both test samples in the present study are similar to or slightly higher than that observed in predecessor studies (BSP107 [1] and BSP116 [5]).

4.2. Continuity between BRR batches

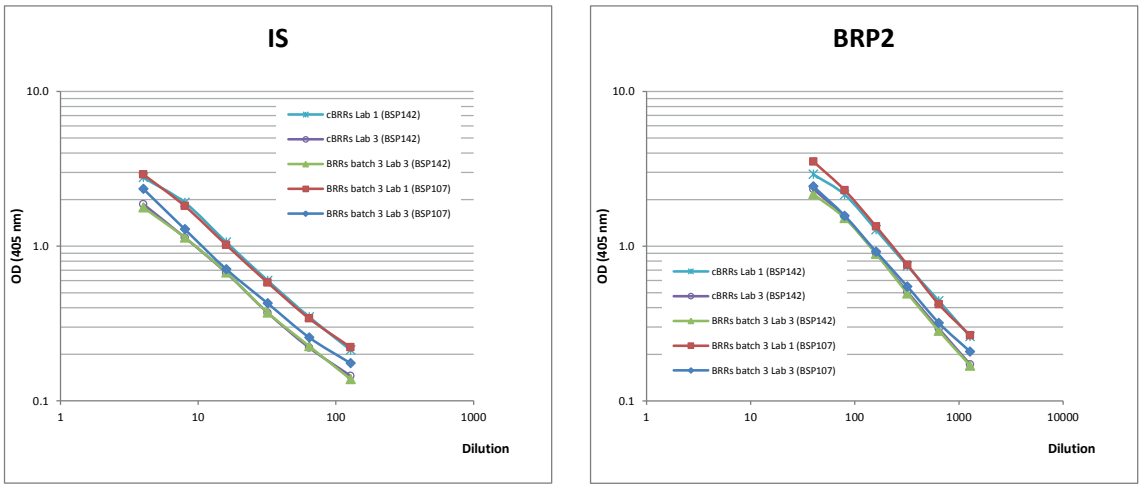
Assessment of continuity was performed by comparison of ODmax levels reached and by observation of the concentration-response profiles for both test samples in comparison to previous determinations obtained in the same laboratories during predecessor studies (, Figure 2).

Table 2 – *Assessment of continuity between batches of detection antibodies (means of 3 assays)*

Sample	Dilution	BSP 107		BSP 142		
		BRRs batch 3 Lab 1*	BRRs batch 3 Lab 3	BRRs batch 3 Lab 3	cBRRs Lab 1	cBRRs Lab 3
IS	1/4	2.912	2.343	1.778	2.776	1.868
	1/8	1.818	1.287	1.137	1.914	1.138
	1/16	1.019	0.710	0.677	1.062	0.673
	1/32	0.581	0.427	0.372	0.600	0.371
	1/64	0.341	0.257	0.226	0.349	0.221
	1/128	0.222	0.175	0.138	0.213	0.145
BRP2	1/40	3.524	2.433	2.168	2.912	2.336
	1/80	2.297	1.571	1.517	2.151	1.547
	1/160	1.340	0.921	0.893	1.276	0.898
	1/320	0.757	0.548	0.495	0.743	0.506
	1/640	0.421	0.319	0.284	0.443	0.293
	1/1280	0.265	0.208	0.169	0.260	0.172

* Mean of 2 assays.

Figure 2 – *Comparison of concentration-response curves obtained in BSP107 and BSP142*



The profiles obtained with both test samples, i.e. BRP2 and IS, are almost identical in each individual laboratory. However, in one of the laboratories, OD values are consistently higher than in the other but since calculation of the antigen content is done in relation to a reference standard, determination of titres of samples to be tested should therefore not be affected if OD values are overall slightly higher or lower in one particular laboratory, provided linearity and parallelism are retained.

4.3. Comparison of the calculated BRP2 titres

A summary of results is given in where the p-values for deviations from parallelism and linearity together with the weighted correlation coefficient r (not r^2) are shown. Significant p-values (< 0.05) are printed on a pale blue background and highly significant p-values (< 0.01) on a dark blue background. Correlation coefficients less than 0.99 are printed on a pale blue background and less than 0.98 on a dark blue background. The table also shows the estimated potencies with associated 95 % confidence limits expressed as percentage of the estimated potency. Assay 1 by laboratory 1 and assay 3 by laboratory 2 show significant deviation from linearity and/or parallelism, but could be retained as valid because the correlation coefficient was at least 0.99 [1]. All assays were thus statistically valid and none of the assays exceeded the maximum allowed width of the confidence interval, i.e. 80-125 % [2].

Potency estimates were combined by taking the weighted or semi-weighted geometric mean (GM) per laboratory. The weighted GM was used when the p-value for homogeneity was higher than 0.100; the semi-weighted GM was used for p-value lower than 0.100. To combine the laboratory GMs, the semi-weighted overall GM was calculated as well as the 95 % confidence limits of the overall mean relative to the estimate.

Table 3 – *BRP2 antigen content determined using the BRRs batch 3 and the cBRRs (central calculations)*

cBRRs												
Lab	Assay	Corr.	Lin.	Par.	Valid?	Est.	Conf. limits	Homog.	Est.	Conf. limits	Homog. (overall)	Overall est.	Overall conf. limits
1	1	0.997	0.000	0.019	Yes	1407	(97 %-103 %)	0.001	1318	(94 %-107 %)	0.000	1360	(95 %-106 %)
	2	0.988	0.988	0.766	Yes	1280	(90 %-111 %)						
	3	0.997	0.765	0.191	Yes	1203	(92 %-109 %)						
2	1	0.999	0.980	0.942	Yes	1301	(97 %-103 %)	0.634	1298	(98 %-102 %)			
	2	0.999	0.854	0.252	Yes	1288	(98 %-103 %)						
	3	0.998	0.958	0.001	Yes	1314	(97 %-104 %)						
3	1	0.999	0.983	0.090	Yes	1433	(97 %-103 %)	0.014	1479	(96 %-105 %)			
	2	0.997	1.000	0.622	Yes	1441	(91 %-111 %)						
	3	0.989	0.991	0.247	Yes	1566	(95 %-105 %)						

BRRs batch 3										
Lab	Assay	Corr.	Lin.	Par.	Valid?	Est.	Conf. limits	Homog.	Est.	Conf. limits
3	1	0.999	0.754	0.265	Yes	1478	(97 %-103 %)	0.000	1474	(95 %-106 %)
	2	0.998	1.000	0.223	Yes	1373	(96 %-105 %)			
	3	0.997	0.998	0.164	Yes	1592	(94 %-106 %)			

Conf. limits = 95 % Confidence limits expressed as percentage of the estimated potency; Corr. = Weighted correlation coefficient; Homog. = p-value for homogeneity; Lin. = p-value for non-linearity; Par. = p-value for non-parallelism.

Based on the results of the establishment study (BSP116), the BRP2 was assigned an antigen content of 1350 IU/mL. shows that, regardless of the BRRs used, the estimated titres of BRP2 are very consistent between studies, with an inter-study mean of 1 371 IU/mL, and low inter-study variation (GCV = 5.2 %).

Table 4 – *BRP2 titre (IU/mL) measured over several studies with BRRs batch 3 and the cBRRs*

Study	Potency (IU/mL)
Current study cBRRs	1360
Current study BRRs batch 3	1474
BSP107	1305
BSP116 (calibration study)	1351
Overall GM	1371
Overall GCV (%)	5.2

5. DISCUSSION

The aim of this study was to confirm the suitability of the new candidate BRR materials (i.e. the primary and secondary detection antibodies cBRRs batch 4) to be used for the *in vitro* determination of antigen content of hepatitis A vaccines using working dilutions defined in a preliminary study using the Ph. Eur. standard method [2]. Comparison of concentration-response curves clearly indicated that the optimal working dilutions should be 1:500 for the new MAbs coupled to 1:400 for the new HRPO-GAM.

To ensure that no laboratory-specific bias had been introduced during the preliminary experiments carried out in 1 single laboratory, a small collaborative study was organised with 3 experienced participating laboratories. The concentration-response curves obtained during the study were compared to those obtained during the prequalification tests. A good consistency of OD values between laboratories and assays was observed, with ODmax reaching levels

consistent with those observed during the predecessor studies [1, 5], and the concentration-response curves also show very similar profiles. Moreover, the BRP2 potency calculated relative to the IS was consistent between studies, laboratories and BRR batches, and close to its assigned potency (1 350 IU/mL).

6. CONCLUSIONS

The results of the study showed that the working dilutions defined for the cBRRs led to similar ranges of OD values and to potencies very close to those observed with previous batches, confirming thereby the suitability of the cBRRs in this setup. It was therefore proposed to establish the new batches of detection antibodies as Anti-hepatitis A virus primary detection antibody BRR batch 4 and Conjugated secondary detection antibody BRR batch 4, with recommended working dilutions of 1:500 and 1:400 respectively. This proposal was adopted by the Ph. Eur. Commission at its 158th session in June 2017. The reference reagents are available from the EDQM as Hepatitis A vaccine ELISA detection antibodies set BRR batch 4 (containing one vial of each detection antibody) under catalogue number Y0001623.

The stability of these detection reagents had been shown previously to be very good at −20 °C [1], therefore no stability study was deemed necessary. The BRRs will thus be stored and distributed to users at this temperature. Their activity will nevertheless be monitored at regular intervals throughout their lifetime.

7. ACKNOWLEDGEMENTS

The organisers wish to express their sincere thanks to Dr S. Morgeaux for her role as project leader for this study, and to all the participants and their colleagues/co-workers at the bench for their contribution to this study. Ms Sally Woodward and Ms Sandra Fromweiler are warmly acknowledged for their skillful assistance throughout the study.

This study was run by the EDQM in the framework of the Biological Standardisation Programme, a joint programme carried out with funding by the European Union and by the Council of Europe, under project code BSP142. This document was produced with financial assistance by the European Union. The views expressed herein can in no way be taken to reflect the official opinion of the European Union.

8. PARTICIPANTS (IN ALPHABETICAL ORDER BY COUNTRY)

Dr E. Dubois, WIV-ISP, Belgium
Dr S. Morgeaux, A. Koy, I. Manniam, P. Variot, ANSM, France
Dr C. Göpfert, PEI, Germany

9. ABBREVIATIONS

ANSM: Agence Nationale de Sécurité du Médicament et des Produits de Santé; BRP: Biological Reference Preparation; BRR: Biological Reference Reagent; BSA: Bovine Serum Albumin; BSP: Biological Standardisation Programme; cBRR: candidate BRR; EDQM: European Directorate for the Quality of Medicines & HealthCare; ELISA: Enzyme-Linked Immuno-Sorbent Assay; GCV: geometric coefficient of variation; GM: geometric mean; HAV: hepatitis A vaccine; HRPO-GAM: Horse Radish Peroxidase-labelled goat anti-mouse antibodies; IS: International Standard; IU: International Unit; Lab: Laboratory; MAbs: monoclonal antibodies; NIBSC: National Institute for Biological Standards and Control; OD: Optical Density; ODmax: maximal OD; OMCLs: Official Medicines Control Laboratories; PBS: phosphate buffered saline; PBS-B-T: PBS-BSA-Tween; Ph. Eur.: European Pharmacopoeia; SD: standard deviation; WHO: World Health Organization.

REFERENCES

[1] Morgeaux S, Variot P, Daas A, *et al.* Validation of a new ELISA method for *in vitro* potency testing of hepatitis A vaccines. *Pharmeur Bio Sci Notes* 2013 (1): 64-92.

[2] *Assay of hepatitis A vaccine*, general chapter 2.7.14. Ph. Eur. 8th Edition. Strasbourg, France: Council of Europe; 2015.

[3] Morgeaux S, Manniam I, Variot P, *et al.* Establishment of hepatitis A detection antibodies set BRR batch 3 for antigen content determination by ELISA. *Pharmeur Bio Sci Notes* 2015: 235-45.

[4] Wood D, Sands D, Heath A. A WHO and Ph. Eur. Collaborative Study of Standards for Inactivated Hepatitis A Vaccine. Ref: BS/99.1914. WHO Expert Committee on Biological Standardization; 1999.

[5] Morgeaux S, Manniam I, Variot P, *et al.* Establishment of hepatitis A vaccine (inactivated, non-adsorbed) BRP batches 2 and 3. *Pharmeur Bio Sci Notes* 2015: 118-30.

APPENDIX

Individual ODs of concentration-response curves as reported by participants

Individual ODs of concentration-response curves obtained with the cBRRs and the BRRs batch 3 during the present study, using the IS or BRP2 as test samples. The IS and BRP2 were pre-diluted 1/4 and 1/40 respectively before loading onto the plates. Hereunder only the dilution on plates is stated.

cBRRs										
Lab	Sample	Dilution	Assay 1		Assay 2		Assay 3		Mean	SD
			Repl 1	Repl 2	Repl 1	Repl 2	Repl 1	Repl 2		
1	IS	1/1	2.537	2.513	3.176	2.877	3.058*	2.052*	2.776	0.31
		1/2	1.838	1.823	2.000	1.994	2.136*	1.763*	1.914	0.10
		1/4	1.057	0.980	1.180	1.182	1.051	0.924	1.062	0.10
		1/8	0.661	0.646	0.615	0.607	0.544	0.524	0.600	0.05
		1/16	0.344	0.363	0.375	0.407	0.307	0.298	0.349	0.04
		1/32	0.222	0.217	0.227	0.232	0.189	0.191	0.213	0.02
		1/64	0.158	0.158	0.164	0.160	0.145	0.140	0.154	0.01
	BRP2	1/1	3.033	2.958	3.183	3.188	2.607	2.500	2.912	0.29
		1/2	2.193	2.190	2.197	2.683	1.867	1.774	2.151	0.32
		1/4	1.272	1.329	1.399	1.523	1.114	1.017	1.276	0.19
		1/8	0.802	0.867	0.646	0.817	0.674	0.653	0.743	0.10
		1/16	0.467	0.432	0.454	0.493	0.419	0.395	0.443	0.04
		1/32	0.278	0.271	0.243	0.285	0.240	0.242	0.260	0.02
		1/64	0.180	0.186	0.184	0.185	0.164	0.177	0.179	0.01
		blank	0.085		0.076		0.078		0.080	
2	IS	1/1	2.185	2.078	2.393	2.419	2.181	2.121	2.229	0.14
		1/2	1.320	1.284	1.477	1.509	1.207	1.276	1.345	0.12
		1/4	0.763	0.722	0.927	0.881	0.731	0.746	0.795	0.09
		1/8	0.432	0.420	0.583	0.514	0.428	0.424	0.467	0.07
		1/16	0.265	0.258	0.376	0.324	0.263	0.262	0.291	0.05
		1/32	0.199	0.178	0.246	0.208	0.181	0.181	0.199	0.03
		1/64	0.184	0.185	0.192	0.160	0.144	0.146	0.169	0.02
	BRP2	1/1	2.505	2.493	2.739	2.808	2.534	2.423	2.584	0.15
		1/2	1.582	1.581	1.831	1.827	1.608	1.524	1.659	0.13
		1/4	0.927	0.906	1.061	1.093	0.979	0.956	0.987	0.07
		1/8	0.532	0.525	0.620	0.632	0.591	0.553	0.576	0.05
		1/16	0.322	0.321	0.377	0.389	0.364	0.335	0.351	0.03
		1/32	0.095*	0.095*	0.239	0.235	0.239	0.221	0.234	0.01
		1/64	0.216*	0.218*	0.179	0.189	0.176	0.162	0.176	0.01
		blank	0.098		0.103		0.092		0.097	
3	IS	1/1	1.954	1.902	1.704	1.555	2.136	1.957	1.868	0.21
		1/2	1.145	1.173	1.004	0.973	1.260	1.270	1.138	0.13
		1/4	0.606	0.662	0.614	0.556	0.790	0.809	0.673	0.10
		1/8	0.373	0.377	0.305	0.293	0.457	0.422	0.371	0.06
		1/16	0.221	0.230	0.189	0.171	0.253	0.259	0.221	0.03
		1/32	0.135	0.146	0.149	0.128	0.157	0.157	0.145	0.01
		1/64	0.106	0.105	0.109	0.102	0.116	0.118	0.109	0.01
	BRP2	1/1	2.348	2.351	2.149	1.836	2.595	2.734	2.336	0.32
		1/2	1.565	1.478	1.401	1.274	1.726	1.838	1.547	0.21
		1/4	0.934	0.866	0.785	0.698	1.051	1.051	0.898	0.14
		1/8	0.527	0.514	0.433	0.372	0.581	0.609	0.506	0.09
		1/16	0.306	0.293	0.237	0.217	0.343	0.361	0.293	0.06
		1/32	0.173	0.171	0.151	0.145	0.199	0.194	0.172	0.02
		1/64	0.125	0.117	0.113	0.115	0.131	0.132	0.122	0.01
		blank	0.071		0.081		0.072		0.075	

* Participants reported a technical problem with these wells. They were excluded from the descriptive statistics.
Repl: replicate; SD: Standard deviation.

BRRs batch 3										
Lab	Sample	Dilution	Assay 1		Assay 2		Assay 3		Mean	SD
			Repl 1	Repl 2	Repl 1	Repl 2	Repl 1	Repl 2		
3	IS	1/1	2.052	1.939	1.524	1.603	1.746	1.803	1.778	0.20
		1/2	1.252	1.237	0.991	1.012	1.133	1.198	1.137	0.11
		1/4	0.764	0.704	0.574	0.562	0.707	0.752	0.677	0.09
		1/8	0.409	0.398	0.319	0.324	0.396	0.385	0.372	0.04
		1/16	0.244	0.249	0.189	0.195	0.235	0.245	0.226	0.03
		1/32	0.15	0.139	0.13	0.129	0.139	0.143	0.138	0.01
		1/64	0.111	0.104	0.103	0.1	0.103	0.104	0.104	0.00
	BRP2	1/1	2.438	2.446	1.902	1.785	2.312	2.122	2.168	0.28
		1/2	1.691	1.678	1.303	1.229	1.561	1.641	1.517	0.20
		1/4	0.959	0.967	0.728	0.722	0.988	0.991	0.893	0.13
		1/8	0.551	0.556	0.395	0.378	0.538	0.552	0.495	0.08
		1/16	0.316	0.306	0.233	0.225	0.305	0.317	0.284	0.04
		1/32	0.178	0.177	0.150	0.143	0.180	0.188	0.169	0.02
		1/64	0.130	0.123	0.109	0.106	0.128	0.130	0.121	0.01
		blank	0.071		0.081		0.072		0.075	

Repl: replicate; SD: Standard deviation.

Replacement, Reduction, Refinement

Animal welfare progress in European Pharmacopoeia monographs: activities of the European Pharmacopoeia Commission from 2007 to 2017

Catherine Lang, Olga Kolaj-Robin, Gwenaël Cirefice, Laure Taconet, Ellen Pel, Sébastien Jouette, Mihaela Buda, Catherine Milne, Emmanuelle Charton[1]

ABSTRACT

Since the opening for signature of the European Convention for the Protection of Animals Used for Experimental and Other Scientific Purposes in 1986, the European Pharmacopoeia Commission and its experts have carried out a programme of work committed to Replacing, Reducing and Refining (3Rs) the use of animals for test purposes. While updates on achievements in the field of the 3Rs are regularly provided, this article summarises the activities of the Ph. Eur. Commission in this field within the last decade.

KEYWORDS

Replacing, reduction and refining, 3Rs, animal welfare, animal tests, *in vivo* method replacement, European Pharmacopoeia, European Pharmacopoeia Commission.

1. INTRODUCTION

The European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS No. 123) of the Council of Europe was opened for signature on 18 March 1986 [1]. This marked the beginning of an intensification of the activities of the European Pharmacopoeia Commission (Ph. Eur. Commission) to review all animal tests in monographs. Ph. Eur. texts are being continuously reviewed with a view to applying the precepts of the Convention for the '**3Rs**' in the use of animals for test purposes:

- **R**eplacement (animals are no longer used for the test)
- **R**eduction (fewer animals are used to achieve the defined aim of the test)
- **R**efinement (a test that causes less distress to the animals used is carried out).

In addition to the traditional 3Rs, the Ph. Eur. Commission has employed 'Removal', a fourth 'R', as a strategy to end the unnecessary use of animals. This involves the removal of the need for regular performance of an animal test that, after scientific scrutiny, has proved to be no longer relevant and can be deleted without replacement with another test.

A review of the achievements of the Ph. Eur. Commission in the field of 3Rs since the elaboration of the Convention has previously been published [2] and relevant information is also available on the website of the European Directorate for the Quality of Medicines & HealthCare

1 European Directorate for the Quality of Medicines & Healthcare, Council of Europe, 67081 Strasbourg, France.

(EDQM)[2] and in other publications [3]. This article provides further updates in this field. In addition to legal aspects related to the 3Rs (section 2), it describes the progress made within the last decade in Ph. Eur. texts and the challenges lying ahead (section 3 and tables in the Appendix), and the tools utilised in the implementation of the 3R principles (sections 4-6). It concludes with perspectives for the future (section 7).

2. DIRECTIVE 2010/63/EU

Directive 2010/63/EU on the protection of animals used for scientific purposes [4], which took full effect on 1 January 2013, replaced Directive 86/609/EEC adopted shortly after European Convention ETS No. 123 [1]. It reinforced the 3R principles and introduced tools such as severity classification to estimate the levels of pain, suffering, distress and lasting harm that are inflicted on the animals. Most importantly, Article 13 (Choice of methods) of the directive has a significant impact for users of the Ph. Eur. as it includes the following statement:

> Without prejudice to national legislation prohibiting certain types of methods, Member States shall ensure that a procedure is not carried out if another method or testing strategy for obtaining the result sought, not entailing the use of a live animal, is recognised under the legislation of the Union.

Application of the quality requirements of the Ph. Eur. is prescribed in EU legislation [5, 6] and thus can be considered as recognised in the context of Article 13. Further considerations on the potential impact of Directive 2010/63/EU on the implementation of the Ph. Eur. can be found in section 5.2.

3. ACHIEVEMENTS OVER THE LAST DECADE

The Ph. Eur. Commission has taken a multi-layered approach to advance 3Rs improvements. This approach begins with the overarching principles, includes the assessment of strategies applicable across different sectors and is anchored in specific texts in individual monographs which directly reduce, replace, refine or remove the use of animals. The main achievements of the Ph. Eur. Commission in this field over the last decade are described below, while detailed information by class of product on specific texts is provided in the Appendix. Despite the many advances, numerous challenges remain and details of some of those encountered during the last decade are also discussed below.

3.1. Overarching principles

3.1.1. Compliance via validated alternatives

As stated in the General Notices (chapter 1) of the Ph. Eur., the methods described in Ph. Eur. monographs are reference methods, essential in case of dispute. Compliance is requested, but alternative methods may be used as long as they lead to the same pass/fail result. In other cases, a detailed validated procedure may be given as an example of a suitable method, meaning that other methods could be used instead without having to demonstrate their equivalence to the example method.

Moreover, the General Notices state that compliance with Ph. Eur. requirements does not imply that performance of all the tests in a monograph is necessary provided the product would comply if tested. In other words, through its General Notices, the Ph. Eur. already allows flexibility in the application of testing requirements.

3.1.2. Consistency of production

The Ph. Eur. also provides flexibility with respect to test performance in the *Demonstration of compliance with the Pharmacopoeia* section in the General Notices:

2 https://www.edqm.eu/en/alternatives-animal-testing.

> Reduction of animal testing: the European Pharmacopoeia is dedicated to phasing out the use of animals for test purposes, in accordance with the 3Rs (Replacement, Reduction, Refinement) set out in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. In demonstrating compliance with the Pharmacopoeia as indicated above […], manufacturers may consider establishing additional systems to monitor consistency of production. With the agreement of the competent authority, the choice of tests performed to assess compliance with the Pharmacopoeia when animal tests are prescribed is established in such a way that animal usage is minimised as much as possible.

The concept of waiving tests as part of a strategy for monitoring consistency of production was added to the general monograph *Vaccines for veterinary use (0062)* as well as to three veterinary vaccine monographs, *Canine leptospirosis vaccine (inactivated) (0447)*, *Bovine leptospirosis vaccine (inactivated) (1939)* and *Infectious bovine rhinotracheitis vaccine (inactivated) (2674)* (9th Edition, January 2017). Omission of tests is therefore possible when consistency is demonstrated and in agreement with the competent authority. It is important to note that compliance with the tests described in Ph. Eur. monographs (during production or for the final lot) is usually not sufficient to ensure consistency of production: suitable additional tools such as statistical process control should also be used.

3.1.3. Substitution of in vivo *tests*

Pharmacopoeia monographs are public standards and are intended to provide quality requirements applicable to all products on the market. Application of the 3Rs to animal testing in existing monographs has been seen to require development of an alternative method applicable, without modification, to all existing products. For finished products, notably vaccines, this aim has rarely been achieved in a way that leads to direct application of the 3Rs. The existing products were developed at a time when the animal model was the standard method, despite the associated relatively high variability, and the products were necessarily developed in such a way as to comply with these models. Demonstration of equivalence of an alternative method to the animal model is not simply problematic, in many instances it may also be of limited relevance. This implies that a complete re-evaluation of the aims of the new test needs to be made to define the relevant aspects that must be validated.

With these difficulties in mind, and to facilitate the transition from *in vivo* to *in vitro* methods, the Ph. Eur. Commission developed a new general chapter on the *Substitution of* in vivo *method(s) by* in vitro *method(s) for the quality control of vaccines (5.2.14)* published in Supplement 9.3 (implementation date January 2018). This provides guidance on how to validate alternative *in vitro* methods in scenarios where a direct head-to-head comparison with an existing *in vivo* method is not possible. The general chapter envisages the possibility that the validity of an alternative *in vitro* method can be demonstrated without such a head-to-head comparison (concept of 'substitution') and discusses alternative approaches for *in vivo* method replacement. Specific recommendations on the substitution of *in vivo* potency and safety tests, together with examples, are provided. A cross-reference to Chapter *5.2.14* has been added to the general monographs *Vaccines for human use (0153)* and *Vaccines for veterinary use (0062)* to increase stakeholder awareness of this important text, which provides additional tools for the efforts to reduce animal testing and encourage the use of alternatives.

3.2. Applicability across sectors

3.2.1. Abnormal toxicity

The abnormal toxicity test (ATT) was originally developed in the early 1900s as a safety test intended to detect extraneous contaminants in biological products. The test is based on the injection of the product to be tested into mice/guinea pigs. The product passes the test if no animal shows any sign of illness, relevant body weight changes, or dies within a defined timeframe. The number of animals used in the ATT has been considerable (e.g. 5 mice and 2 guinea pigs are used for each vaccine batch to be tested), making the ATT one of the most controversial animal tests in the Ph. Eur., and therefore a priority target for replacement.

After a review of historical batch data in 1999, references to the ATT were removed from the Tests section of over 80 monographs and replaced by a statement in the Production section, which prescribed that the manufacturing method be validated in such a way as to ensure that the product would comply with the test if it were performed. As a result of this exercise, the ATT was no longer required to be performed routinely on each batch (deletion of the test as a routine batch release test) and had to be performed during product development only.

The relevance of the ATT was reviewed in depth during a European Partnership for Alternative Approaches to Animal Testing (EPAA) International Workshop in September 2015 [7]. The test was considered to be outdated and shown to be 'neither specific, reproducible, reliable nor suitable for the intended purpose'. Additionally, with modern manufacturing and stringent quality measures in place to control and detect contaminants, it has also become unnecessary. It was concluded that the ATT lacked scientific relevance and that its omission would not compromise the safety of biologicals.

Based on this detailed review, the Ph. Eur. Commission deleted the ATT from 49 Ph. Eur. monographs encompassing areas such as vaccines and immunosera for human use, biotherapeutics, allergens, antibiotics/antimycotics and plastic containers. Revised monographs omitting the ATT from the Production section will be included in Supplement 9.6 (January 2019) with simultaneous suppression from the Ph. Eur. of general chapter *Abnormal toxicity (2.6.9)*, as it will no longer be referred to in any monograph and will thus be obsolete. With the publication of Supplement 9.6, the complete suppression of the ATT from the Ph. Eur. will have been achieved.

3.2.2. Rabbit pyrogen test (RPT)

About 60 Ph. Eur. texts still refer to the pyrogen test (*Pyrogens 2.6.8*). Among these are monographs on vaccines for human use, blood products, antibiotics, solutions for dialysis or organ preservation, and general chapters on plastic containers for blood, syringes and sets for transfusion. The Ph. Eur. Commission is making every effort to proceed with the replacement of this widely used animal test. Typically, where possible, the pyrogen test has been replaced by the bacterial endotoxin test (BET). In a recent revision of the chapter *Guidelines for using the test for bacterial endotoxins (5.1.10)* (Supplement 8.8, July 2016), a recommendation has been introduced to perform a risk assessment when using the bacterial endotoxin test as a pyrogenicity test to confirm the absence of potential contamination by non-endotoxin pyrogens. The *Monocyte-activation test (2.6.30)* is a suitable method for ruling out the presence of non-endotoxin pyrogens in substances or products at release or during the production process (see section 3.2.3).

3.2.3. Monocyte activation tests (MAT)

The monocyte activation test (MAT) is an *in vitro* test which can be used to replace the RPT after suitable validation has been performed. It is used to detect or quantify substances that activate human monocytes to release endogenous mediators such as pro-inflammatory cytokines (e.g. TNFα, IL-1β, IL-6). The monocyte source used in the test can be whole blood or peripheral blood mononuclear cells (PBMC) from a single or several donors after pooling, with or without cryopreservation storage. Moreover, monocytic continuous cell lines are also available for this test. The general chapter *Monocyte-activation test (2.6.30)* was introduced in Supplement 6.7 (April 2010) following a recommendation resulting from a European Centre for the Validation of Alternative Methods (ECVAM) Workshop [8]. While the MAT offers significant improvements in terms of 3Rs, it had been reported that the test had not been used as widely as expected for quality control purposes since its introduction in the Ph. Eur. As a result, the EDQM carried out a survey in 2013 with the intention of improving the technical content of the chapter, and a revised version of the chapter was published in Supplement 9.2 (July 2017). A key challenge that remains for the EDQM is to be able to provide a reference standard for a non-endotoxin pyrogenic substance. This is now being addressed via a joint project run by the World Health Organization (WHO) and the EDQM Biological Standardisation Programme (BSP) (BSP149) to establish a reference material suitable for this purpose.

The general chapter *Pyrogens* (*2.6.8)* was revised in Supplement 8.8 (July 2016) with the addition of a statement encouraging the replacement of the RPT with the MAT:

> In accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, tests must be carried out in such a way as to use the minimum number of animals and to cause the least pain, suffering, distress or lasting harm. Wherever possible and after product-specific validation, the pyrogen test is replaced by the monocyte-activation test *(2.6.30)*.

Monographs and general chapters which refer to the pyrogen test typically do not refer to the MAT: the reference is made only once in general chapter *2.6.8*. Once a full validation package for the individual substance/preparation/material in question has been made available, replacement of the RPT with the MAT could be envisaged.

3.3. Specific improvements to individual monographs/chapters

Descriptions and details of each revision of Ph. Eur. texts can be found in the EDQM Knowledge Database under 'view history'.

3.3.1. Vaccines for veterinary use

In the absence of a suitable *in vitro* alternative, a first approach to address the 3Rs when a method involving significant suffering or distress of animals (e.g. an LD50 assay) is prescribed in the Ph. Eur. has been to introduce 'humane' end-points. In this spirit, the Ph. Eur. monograph *Rabies vaccine (inactivated) for veterinary use (0451)* was revised in Supplement 6.1 (April 2008). The revision introduced a new section on alternative (i.e. non-lethal) end-points, describing early clinical signs of rabies infection that can be observed and used as an alternative end-point in the potency assay, together with a score chart. Analysts are expected to 'validate' the use of clinical signs as end-points (i.e. show that the use of such alternative end-points yields assay results equivalent to those obtained when a lethal end-point is used) by scoring a sufficient number of batches using both the clinical signs and lethal end-points. Since the test is carried out routinely for the release of vaccine batches, manufacturers have the opportunity to incorporate the alternative scoring without having to perform separate tests for validation.

As of Supplement 7.7 (April 2013), following the decision of the Ph. Eur. Commission, the Target Animal Batch Safety Test (TABST) was deleted from the Ph. Eur. This deletion of the TABST goes a step further than the option, available since 2004, of waiving the use of the TABST for established vaccines. This decision was based on a number of parameters, including poor sensitivity of the test, a very limited number of batches failing the test and observations of field safety issues with batches compliant with the TABST. Taking into account new developments (e.g. improvements in the manufacturing process of veterinary vaccines in recent decades and the introduction of new requirements regarding in-process testing and control of the starting materials), the risk/benefit ratio no longer supported retention of such a test for routine batch release and it was therefore decided to delete it (see [9] and [10] for more details). This change has already greatly reduced the number of animals used for the control of veterinary vaccines, while maintaining the level of safety.

Several recent 3Rs-related amendments in relation to veterinary vaccines were introduced at the same time (9th Edition, January 2017) (see Appendix, Table 1):

- manufacturers were encouraged to use modern *in vitro* methods, such as *Nucleic acid amplification techniques (2.6.21)*, instead of the test for antibody induction in animals to identify inactivated vaccines. According to the general monograph *Vaccines for veterinary use* (*0062*), for inactivated vaccines, the identification test may be combined with the potency test to reduce the number of animals used;
- thanks to the elaboration of a new chapter *Healthy chicken flocks for the production of inactivated vaccines for veterinary use (5.2.13),* which sets quality requirements that provide guarantees with regard to contamination by extraneous agents, together with appropriate validation of the inactivation process (done once in the lifetime of the vaccine),

it was possible to omit the test for specified extraneous agents performed on the final product. The introduction of a reference to chapter *5.2.13* containing requirements for healthy flocks rendered the test for specified extraneous agents previously performed on each final product obsolete, and allowed the deletion of the specified extraneous agents test (using either 10 chickens or 2 pigs) for 6 veterinary vaccine monographs (*0870*, *0959*, *0960*, *0963*, *1202* and *1392*);

- an *in vitro* batch potency test for Leptospira vaccines was introduced;
- a serological assay for rabies vaccines was introduced following the completion of BSP105 ([11]; see also section 4).

Some improvements were also made when the regulatory requirements were reviewed, such as updating the *Infectious chicken anaemia vaccine (live) (2038*) monograph immunogenicity test to replace solitary housing of laying hens and young chickens with social housing in stable groups, which is less stressful for the animals.

Where, in spite of all the efforts made by the Ph. Eur. Commission to promote animal welfare, animal tests still subsist in routine testing, 'door openers' may have been included in Ph. Eur. texts. These indicate that alternative methods can replace the animal test and are to be seen as encouragement for manufacturers to develop their own alternative method. A reference to the type of alternative method may be given as an example. To illustrate this, see the example of *Canine leptospirosis vaccine (inactivated) (0447)* (Ph. Eur. 9th Edition, January 2017) for which a single, universal alternative method could not be developed due to the complexity of the vaccines, but which nevertheless includes as first option a suitably validated *in vitro* batch potency test able to determine the content of one or more antigenic components which are indicators of protection and which are specific for that serovar. This does not preclude manufacturers from developing other types of more appropriate alternative methods (for example, where new techniques are available).

3.3.2. Vaccines for human use

The Ph. Eur. monograph on *Rabies vaccine for human use prepared in cell cultures (0216)* was revised in Supplement 6.1 (April 2008). In the same way as for the monograph *Rabies vaccine (inactivated) for veterinary use (0451)*, and to foster the use of humane end-points in the potency assay, the revision introduced a new section describing early clinical signs of rabies infection that can be observed and used as an alternative end-point, together with a score-chart (see section 3.3.1 for further details). A new general chapter on *Residual pertussis toxin and irreversibility of pertussis toxoid (2.6.33)* was included in Supplement 7.8 (July 2013). It introduced a standard protocol for the Histamine Sensitisation Test in mice (HIST), based on the outcome of a collaborative BSP study (BSP076), with the intention of facilitating the standardisation of the method and therefore reducing the unnecessary use of animals [12]. Based on the results of a more recent collaborative study (BSP114) completed in 2015 [13], the introduction of an *in vitro* Chinese Hamster Ovary (CHO) cell-clustering assay for the determination of residual pertussis toxin as a replacement for the animal HIST is under discussion. The removal of the test for irreversibility of pertussis toxoid has also been proposed.

In vitro methods have, in a number of cases, been introduced as an alternative to or replacement for *in vivo* testing. One example was the introduction in the general chapter *Assay of hepatitis A vaccine (2.7.14)* of a validated ELISA method for determination of the antigen content [14] as an alternative to the serology assay in mice, a change included in Supplement 8.5 (July 2015). Various strategies have also been used to promote both the reduction and refinement of *in vivo* assays, for example through the introduction of serology assays as an alternative to lethal challenge methods for diphtheria, tetanus, acellular pertussis and rabies vaccines; after sufficient experience is gained, these may be used as a simplified model (e.g. a single-dilution model), leading to a reduction in the number of animals needed [15-17].

More recently the Ph. Eur. Commission's attention turned to the current requirements for extraneous agent testing. The aim was to rationalise these requirements without in any way

compromising safety. As part of this initiative, the general chapters *Tests for extraneous agents in viral vaccines for human use (2.6.16)* and *Cell substrates for the production of vaccines for human use (5.2.3)* were revised in Supplement 9.3 (January 2018). The revised general chapter *2.6.16* recommends that the testing strategy for extraneous agents should be established based on a risk assessment and the list of tests must be adapted depending on the extraneous agents that have the potential to contaminate the product. Molecular biology methods may be considered for the detection of specific viruses and/or for the broad detection of viruses. As part of the revisions of both general chapters, the tests in adult mice and guinea pigs were deleted as they were considered redundant due to the presence of other tests providing risk mitigation. In addition, the tests in suckling mice and control eggs are now to be used only if a risk assessment indicates that the tests can provide risk mitigation.

3.3.3. Blood products

A systematic review of animal tests prescribed in monographs for medicinal products derived from human blood and human plasma (hereinafter called 'plasma-derived products') has been undertaken with a view to introducing, wherever possible, provision for the use of an *in vitro* method. This work has been driven by consultations with stakeholders; it is based on the outcome of the EDQM internal workshop on the *in vitro* pyrogen test (2005) and of a survey conducted by the EDQM in 2005 to gather data on the application of different test methods for the replacement of the RPT for plasma-derived products. Notably, data demonstrating equivalency of test methods has enabled the revision of 15 monographs to allow a validated *in vitro* test, such as the bacterial endotoxin test, to be used as an alternative to the RPT (see Appendix, Table 3). Typical wording in monographs is as follows:

> *Pyrogens (2.6.8)* or *Bacterial endotoxins (2.6.14)*. It complies with the test for pyrogens or, preferably and where justified and authorised, with a validated *in vitro* test such as the bacterial endotoxin test.

In addition, the European Medicines Agency (EMA) has revised its *Guideline on plasma-derived medicinal products* [18], by introducing a cross-reference to the *Guideline on the replacement of rabbit pyrogen testing by an alternative test for plasma-derived medicinal products* [19]. The revised monographs together with the EMA guideline constitute a powerful combination of tools that will help users to implement a replacement for the RPT.

As a result of the Ph. Eur. Commission's efforts, the majority of Ph. Eur. monographs on plasma-derived products currently promote the use of non-animal alternatives. The Ph. Eur. is continuing to examine whether alternatives to the RPT are available for the remaining monographs, and to complete the 3Rs-driven revision process.

3.3.4. Biological and biotechnological substances

A number of actions had already been undertaken to replace and reduce the use of animal testing in the field of biological and biotechnological products [20-25]. The RPT has been replaced by the bacterial endotoxin test in all but one monograph (see section 7.4), while the ATT was recently deleted from four monographs (see Appendix). Finally, substitution of the assay on isolated rat adrenal cells with a liquid chromatography (LC) method in the *Tetracosactide (0644)* monograph in Supplement 6.3 (January 2009) concluded the replacement of *in vivo* bioassays in monographs for synthetic peptides.

3.4. Challenges

The Ph. Eur. Commission is committed to including validated 3Rs methods in specific monographs wherever possible. In order to include such texts in a monograph, however, there are certain prerequisites, based on scientific principles and the applicability of the method to all or most products on the EU market, which must be met. According to the general principles noted above, the absence of a description of an alternative test in the Ph. Eur. does not preclude the possibility to use suitably validated 3Rs alternative methods developed for individual products provided they are approved by the licensing authority (see EMA Guideline on the principles of regulatory acceptance of 3Rs testing approaches [26]).

Some of the efforts already invested to replace the *in vivo* test in the Ph. Eur. monographs that still contain an animal-based potency assay are described below.

3.4.1. Erythropoietin

The *Erythropoietin concentrated solution (1316)* monograph comprises two potency assays carried out in polycythaemic (method A) and in normocythaemic mice (method B). Several attempts to implement the 3Rs principles in this monograph and to replace these *in vivo* assays have been made in the past.

As the biological activity of erythropoietin *in vivo* is known to critically depend on the level of terminal sialyation of the carbohydrate chains, and is therefore quantitatively related to the isoform distribution, the initial step towards the replacement of the animal test in the monograph was to improve the isoform distribution test. Consequently, a collaborative study was carried out to assess a capillary zone electrophoresis (CZE) method for this purpose [27]. Although the CZE method replaced the isoelectric focusing (IEF) test in the 4th Edition of the Ph. Eur. (implementation date January 2002), its introduction did not justify the deletion of the *in vivo* bioassays.

Subsequently, following a proposal from ECVAM's Scientific Advisory Committee, the inclusion of an *in vitro* activity test in addition to the two *in vivo* assays was proposed [28]. The comparative data gathered over the years was expected to facilitate the eventual replacement of the bioassays in the future. However, further to comments received during the public enquiry phase, this proposal was abandoned as it was considered that more knowledge and experience of *in vitro* assays had to be gathered first.

During the subsequent revision of the monograph another recommendation from ECVAM to delete assay method A leaving method B as the only assay for erythropoietin was carefully considered. The sacrifice of a considerable number of animals that would result from a compulsory revalidation of method B by users currently employing method A was judged unacceptable and unnecessary. Therefore, the Assay section has been maintained with two *in vivo* bioassays until the introduction of an *in vitro* assay replacing them can be executed.

In addition to these activities, the European erythropoietin manufacturers had been contacted on a number of occasions, including two surveys performed in 2005 and 2009, for their assistance in replacing the animal assay. Significant data using available validated *in vitro* methods was gathered during the establishment of the most recent batch of Erythropoietin BRP [29] and its analysis is ongoing. In the meantime, in order to avoid overconsumption and frequent replacement of Erythropoietin BRP, which is calibrated in International Units in a bioassay that requires the use of animals, a separate reference standard, Erythropoietin for physico-chemical tests CRS, was established to be used in CZE, polyacrylamide gel electrophoresis and immunoblotting and in peptide mapping identification tests [30].

3.4.2. Follitropin monographs

The *Follitropin (2285)* and *Follitropin concentrated solution (2286)* monographs were published in the 8th Edition of the Ph. Eur. Despite the efforts invested during the drafting phase, no suitable alternative to the *in vivo* potency test could be found. Hence, the monographs in question contain an *in vivo* assay in which the follicle-stimulating activity of follitropin is estimated in rats.

An assessment of IEF and CZE as possible replacements to the follitropin *in vivo* potency assay had been performed in an international collaborative study co-ordinated by ECVAM [31]. However, no correlation with the International Unit could be found and the methods could not be applied to all follitropin products available in Europe.

An authorised IEF-based method developed by a manufacturer as part of a well-controlled process had also been considered as a potential *in vitro* alternative. However, as it was based on a proprietary analysis tool, it could not constitute a Ph. Eur. method.

3.4.3. Radiopharmaceuticals

Many monographs for radiopharmaceutical preparations were elaborated in the 1970s and 1980s. Animal tests, mainly physiological distribution tests, were included to ensure the desired distribution of the radiopharmaceutical preparation in the body. Physico-chemical tests have evolved with time and are often able to control the composition of the radiopharmaceutical preparation; they can thus be used to replace these physiological distribution tests. However, as with other groups of pharmaceuticals, the demonstration of equivalence of potential alternative techniques to the animal tests would require the sacrifice of many animals.

All monographs on radiopharmaceutical preparations that contained a test involving animals were carefully reviewed. For some, for example radiolabelled colloids, it was considered that current physico-chemical methods alone could not provide adequate quality control and the physiological distribution test could not be replaced or deleted. In other cases the preparations concerned were old products of rather minor economic importance and manufacturers would not invest resources to develop and validate methods to replace the animal test. Academia was not interested either, since research was focused on new products. In the cases of *Technetium (^{99m}Tc) medronate injection (0641)* and *Technetium (^{99m}Tc) etifenin injection (0585)* it proved possible to replace the animal test with a combination of physico-chemical tests.

The revised general monograph *Radiopharmaceutical preparations (0125)* (Ph. Eur. 7.5, July 2012) clearly outlines that 'tests involving animals should be avoided wherever possible'. New monographs on radiopharmaceutical preparations do not contain animal tests.

4. BIOLOGICAL STANDARDISATION PROGRAMME OF THE EDQM

The application of the 3Rs principles in the Ph. Eur. has been greatly facilitated by the Biological Standardisation Programme (BSP) of the EDQM [3]. Since its establishment in 1991, this programme has provided the means to carry out studies to develop and validate methods promoting the application of the 3Rs that are subsequently incorporated into the monographs and chapters of the Ph. Eur. The BSP is co-financed by the EU and the Council of Europe.

The BSP establishes Ph. Eur. working standards (mostly biological reference preparations (BRPs)) and fosters method development in the field of biologicals for human and veterinary use with a focus on method validation for 3Rs purposes. It is overseen by a Steering Committee made up of the chairs of the Ph. Eur. groups of experts involved and the chairs of the European Medicines Agency's Biologics Working Party and Immunologicals Working Party, co-opted experts on specific subject areas, a representative from the EU Commission and the EMA, the Director of the EDQM and an observer from WHO. This Steering Committee takes decisions on the programme of activities and at critical stages of individual projects. The goal is to introduce the validated methods and standards into Ph. Eur. monographs. BSP projects take methods that have undergone proof of concept development and validation in a local context (e.g. by an individual Official Medicines Control Laboratory (OMCL)) or through other projects (e.g. those run by EURL-ECVAM, EPAA, VAC2VAC), and where necessary complete the validation package before using large-scale collaborative studies to demonstrate their general suitability in a wider context [32]. To date 22 projects have been initiated in the interest of 3Rs method development.

A number of projects have been completed in the last 10 years. These include validation of 2 *in vitro* assays to completely replace the use of animals in potency testing of human tetanus immunoglobulins [34-35] and an *in vitro* alternative assay for Hepatitis A vaccine potency [14]. A project to standardise a CHO cell-clustering assay for residual pertussis toxin in acellular pertussis vaccines [13] was completed and, together with a decision by the relevant Ph. Eur. group of experts based on pertinent data, has resulted in a proposal for complete removal of the HIST in mice from general chapter *Residual pertussis toxin and irreversibility of pertussis toxoid* (*2.6.33)*. Other completed projects include a serological assay for acellular pertussis vaccines in guinea pigs which can be combined with serological assays for diphtheria and tetanus components in combined vaccines [15-17], as well as a serological assay for the potency of rabies vaccine (inactivated) for veterinary use [11]. There are several ongoing

projects at different stages of completion, such as validation of an *in vitro* assay (BINACLE: *in vitro* binding and cleavage assay) for the detection of tetanus toxin activity in human and veterinary vaccines for tetanus (BSP136), *in vitro* assays for consistency testing of diphtheria and tetanus antigen content/potency in human vaccines (BSP113), an *in vitro* assay for potency of erythropoietin as a follow up to BSP120, the study to establish Erythropoietin BRP batch 4 [29], a serological potency test for whole-cell pertussis vaccines (BSP104), and in collaboration with the EPAA, the validation of an *in vitro* ELISA assay for antigen content/potency of human rabies vaccines (BSP148) and an *in vitro* replacement for the minimum lethal dose (MLD) and the total combining power (TCP) assays for Clostridium septicum vaccine (BSP130).

The BSP remains open to new proposals within the scope of its activity and encourages all stakeholders to make relevant contributions for consideration.

BSP achievements in the field of the 3Rs are also reported on the EDQM website.[3]

5. EDQM CONFERENCES

Over the years, the EDQM has been eager to facilitate the implementation of 3Rs projects by organising international conferences bringing together experts in the field from regulatory authorities, pharmacopoeias, industry and academia as evidenced by the examples below.

5.1. EDQM International Symposium: Alternatives to Animal Testing – New Approaches in the Development and Control of Biologicals, 23-24 April 2008, Dubrovnik, Croatia

Participants acknowledged that considerable progress had been made in setting non-animal requirements, especially in Europe, but that implementation and regulatory acceptance of 3Rs methods were still key elements that needed further work, in particular for routine application in the control of biologicals. Better transparency and dissemination of existing and future scientific work and achievements should be promoted by publication in appropriate journals and the use of other platforms. It was recognised that Europe had taken a leading role in addressing the challenges and was encouraged to continue to promote new ideas and their application. However, the need for international harmonisation was strongly expressed and supported. Representatives from all the European and international institutions present indicated their willingness to investigate means to improve the situation.

5.2. EDQM International Symposium: Alternatives to Animal Testing, 8-9 September 2011, Strasbourg, France

The aim of the symposium was to share information and experiences of the advances that had been made in this field with regard to the EDQM's BSP and the Ph. Eur. Particular attention was given to the successful completion of a number of EDQM collaborative studies for the validation of 3Rs methods in the fields of human and veterinary vaccines and human blood-derived products, as well as to the Ph. Eur. Commission's efforts to replace the RPT. Aimed at facilitating the practical implementation of the new methods, the symposium provided the opportunity for an in-depth discussion of the new methods and also to prepare for the implementation of Directive 2010/63/EU. The symposium was followed by a thorough evaluation of the impact of the Directive. It was concluded that whenever the Ph. Eur. offered the possibility to carry out either an *in vivo* test or an *in vitro* alternative, the use of the *in vitro* alternative would become obligatory in the EU. Ph. Eur. texts that continued to describe animal methods were found to be compatible with the provisions of the Directive.

5.3. EDQM Workshop on Alternatives to the Leptospirosis Batch Potency Test, 26-27 January 2012, Strasbourg, France

As part of the efforts of the Ph. Eur. Commission to replace *in vivo* with *in vitro* methods, and in a bid to develop alternatives to the batch potency test for leptospirosis vaccines, a workshop

3 https://www.edqm.eu/en/BSP-programme-for-3Rs-1534.html.

targeted at leptospirosis vaccine manufacturers discussed (current and future) alternative methods to the hamster potency test for leptospirosis vaccines, with a view to defining a clear strategy for its replacement. The workshop provided the opportunity for an in-depth discussion of alternative methods and their practical implementation. Participants agreed that a single, universal alternative method could not be developed, due to the complexity of the vaccines (relevance of specific antigens as protective agents, number of serotypes, number of serovars, combinations, presence/absence of adjuvants). However, during the workshop it was shown that an alternative method had already been successfully implemented in Europe, approved by a competent authority, with a further example from the US. These methods use lipopolysaccharide (LPS)-based antigen quantification by ELISA. There was unanimous agreement among the participants present that moving towards complete *in vitro* testing for leptospirosis vaccines is possible and should be promoted.

Further to the EDQM workshop, monographs *Bovine leptospirosis vaccine (inactivated) (1939)* and *Canine leptospirosis vaccine (inactivated) (0447)* were revised for the 9th Edition to introduce the possibility of using alternative methods to the method using guinea-pigs (e.g. LPS-based antigen quantification), thereby contributing to animal welfare (3Rs). Manufacturers are encouraged to develop alternative *in vitro* methods to the animal test for batch release (first option of choice) using appropriate tools such as the monitoring of production consistency and appropriate antigen quantification.

5.4. EDQM International Symposium: The Challenges of Quality Requirements for Fish Vaccines, 10-11 May 2016, Oslo, Norway

The symposium was aimed at discussing the current requirements with a focus on alternative methods, already in use or under development, to replace the challenge batch potency test. The audience discussed the possibility of introducing humane end-points in Ph. Eur. monographs for fish vaccines, and to revise the four monographs already published [*Furunculosis vaccine (inactivated, oil-adjuvanted, injectable) for salmonids (1521), Cold-water vibriosis vaccine (inactivated) for salmonids (1580), Vibriosis vaccine (inactivated) for salmonids (1581), Yersiniosis vaccine (inactivated) for salmonids (1950)*]. The potential need for new Ph. Eur. monographs, such as a general monograph dedicated to vaccines intended for fish and individual monographs for fish vaccines, for example for the Mediterranean region or for other fish diseases, was also discussed.

Further to the EDQM International Symposium, fish vaccine monographs *1521*, *1580*, *1581* and *1950* were revised for Supplement 9.2 (July 2017) to clarify that alternative methods are not limited to serological methods.

6. PARTNERSHIP WITH REGULATORY AUTHORITIES AND OTHER ORGANISATIONS

Regulatory authorities and OMCLs from the 38 countries signatory to the Convention on the Elaboration of a European Pharmacopoeia as well as observers are key players in the 3Rs achievements. The Ph. Eur. works closely with them and relies on their expertise and motivation to effect important changes. In addition, the EDQM regularly exchanges information with the EMA through its relevant working parties. Particularly relevant for 3Rs, the EDQM participates as an observer to the Joint Committee for Medicinal Products for Veterinary Use/ Committee for Medicinal Products for Human Use Working Group on the Application of the 3Rs in Regulatory Testing of Medicinal Products (EMA Working Group on the Application of the 3Rs in Regulatory Testing of Medicinal Products – J3RsWG), an Expert Group created in 2010 by the EMA to provide 3Rs advice on scientific/technical matters related to regulatory testing of medicinal products for human and veterinary use.

Through this group, the EMA has published a position on the application of the 3Rs in the testing of medicines, and issued *Recommendations for marketing-authorisation holders on their need to comply with 3Rs methods in the European Pharmacopoeia* [33]. Specific recommendations in line with monograph/chapter revisions have also been established for

hepatitis A vaccine [36], revisions related to extraneous agents testing and cell substrates for vaccines for human use [37], and a series of recommendations on monographs for veterinary vaccines [38, 40] reinforcing the need to apply the 3Rs concept of the Ph. Eur. in a timely manner. This co-operation is an important element in ensuring awareness and implementation of the various 3Rs advances. In addition the EDQM has contributed actively to the activities of the EPAA and interacts with EURL-ECVAM on different topics of common concern.

7. FUTURE PERSPECTIVES

Although significant progress in the field of 3Rs has been achieved over the last 10 years, the Ph. Eur. Commission is aware of the work still to be done and is committed to further incorporating the 3Rs principles in pharmacopoeial texts. Some of the areas identified as a focus for the future are outlined below.

7.1. Vaccines for veterinary use

For veterinary vaccines, further work is ongoing/will be undertaken with a view to replacing as many animal tests as possible, for example:

- Revision of the extraneous agents testing approach to reinforce risk assessment and to be open to any suitable method, including *in vitro* methods; application of the consistency approach to manufacturing, including an overall risk management strategy for starting material and final product testing.
- Deletion of the remaining tests for specified extraneous agents (monographs 0744, 0965, 1953, 1954 and 1943) in light of the new approach for extraneous agents testing described above.
- Revision of the Identification sections for all live vaccine monographs to open to any suitable method. Currently identification is performed with an immunostaining/neutralisation test in cultures (cells or SPF eggs for avian vaccines) using a monospecific antiserum/monoclonal antibodies. A similar exercise had already been performed for all monographs on inactivated vaccines (see section 3.3.1) and is included in the 9th Edition of the Ph. Eur. Moreover, in several recently drafted or revised monographs on inactivated vaccines, reference to the antibody induction test has been removed, thereby allowing the use of any suitable method.
- Revision of the monograph *Clostridium septicum vaccine for veterinary use (0364)* upon finalisation of the BSP study (BSP130) for the replacement of MLD and TCP tests. This exercise may also have an impact on other monographs for toxoids from cytotoxic clostridial toxins (e.g. 0362, 0363).
- Promotion of a move towards *in vitro* methods for the potency testing of fish vaccines.
- The relevance of the test for 'irreversibility of toxoid' described in monograph *Tetanus vaccine for veterinary use (0697)* is under assessment (see section 7.2).

7.2. Vaccines for human use

The potency assays for established human vaccines (e.g. diphtheria and tetanus vaccines) were initially based on lethal challenge methods and were refined over time with the introduction of serology assays and options for single-dilution assays. However, further work is required to achieve complete replacement of these *in vivo* methods by *in vitro* methods and avoid the use of animals for the determination of potency. For rabies vaccines, a lethal challenge method is still described in the monograph as the reference method for potency determination, despite previous initiatives to encourage the use of refined methods based on non-lethal end-points (see 3.3.2). Efforts to validate a suitable *in vitro* assay will be followed closely (see section 4).

The specific toxicity tests applied to vaccines such as tetanus and acellular pertussis vaccines continue to use animals. In this regard, a proposal to remove the test for irreversibility of pertussis toxoid and replace the HIST with a CHO cell-clustering assay in the test for

residual pertussis toxin is being examined (see section 3.3.2). Likewise, the relevance of the test for irreversibility of the tetanus toxoid applied to tetanus vaccines for both human and veterinary use is being questioned and will be further assessed. An endopeptidase assay for determination of tetanus toxin activity (BINACLE) has been developed and is being tested in a collaborative study (BSP136).

Several other BSP projects are under way to advance the development of *in vitro* alternatives to animal methods used for vaccine quality control. These include, but are not limited to, the validation of an antigen content assay for consistency evaluation of diphtheria and tetanus toxoids (BSP113) and the validation of an ELISA potency assay for determination of the potency of human rabies vaccines (BSP148).

7.3. Biological and biotechnological substances

Although a number of attempts have already been made to minimise animal testing and replace the *in vivo* bioassay in the *Erythropoietin concentrated solution (1316)* monograph (see section 3.4.1), introducing an *in vitro* assay alternative has proved difficult to date. One of the reasons is the variable ratios of *in vivo* to *in vitro* bioactivity of erythropoietin from different sources. Moreover, it has not yet been demonstrated that any of the existing cell-based *in vitro* bioassays could be universally applied. Finally, the International Unit of erythropoietin bioactivity, defined by the WHO International Standard, has been established on the basis of and is traceable to the *in vivo* bioassay procedures, further complicating the task of replacement of the *in vivo* assay. Nevertheless, the substantial data on assaying the potency of erythropoietin using both *in vivo* and available *in vitro* assays gathered during recent years constitute a basis for a new discussion.

Similarly, the replacement of the *in vivo* potency assay in follitropin monographs remains a priority. Users are kindly invited to submit any suggestions regarding potential alternative approaches on this topic to the EDQM.

7.4. Test for pyrogens (2.6.8)

In total, 59 Ph. Eur. monographs still refer to the RPT (*2.6.8*). Of those, 2 monographs covering blood products continue to prescribe an RPT as the sole method to test for pyrogens. Similarly, 3 monographs pertaining to containers for pharmaceutical use and 8 antibiotics monographs still refer to an RPT only, while the efforts to remove it from the last RPT-containing monograph on biological and biotechnological substances *(Urokinase 0695)* are ongoing. In the field of human vaccines, 8 monographs continue to only prescribe an RPT, whereas other human vaccine monographs limit the use of this test to the validation of the manufacturing process and whenever revalidation is necessary, through a statement in the Production section.

A review of the need to perform this test will be made and the possibility of replacing it with a specific requirement in the monograph for an appropriate *in vitro* test (e.g. BET or MAT) is envisaged.

8. CONCLUDING REMARKS

As previously [2], the achievements of the Ph. Eur. Commission in the field of animal welfare in the last decade were significant and have had an impact on several hundreds of Ph. Eur. texts. The animal tests that remain in the Ph. Eur., after more than three decades of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS No. 123) of the Council of Europe, are those that are the most difficult to eliminate. The efforts developed to replace, reduce and refine the use of animals in the Ph. Eur. will therefore have to be intensified as the EDQM continues to encourage and support studies that lead to progress in animal welfare. In order to benefit fully from the current achievements, continued collaboration between the EDQM, regulatory authorities and manufacturers is needed to facilitate implementation. The EDQM will also continue to engage and exchange information with partners outside Europe to foster, as far as possible, common approaches and the acceptance of 3Rs advances on a global level.

9. REFERENCES

1. Council of Europe. European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, ETS No. 123; Strasbourg, France 18 March 1986.
2. Castle P. Replacement, Reduction, Refinement (3Rs), Animal welfare progress in European Pharmacopoeia monographs. *Pharmeuropa* 2007; 19 (3): 430-441.
3. Milne C, Buchheit KH. EDQM's activities in the field of quality control of vaccines. Proceedings of the 8th World Congress on Alternatives and Animal Use in the Life Sciences; 2011, Montreal, Canada 2011: 1/12: 65-69.
4. Directive 2010/63/EC of the European Parliament and of the Council of 22 September 2010 on the Protection of Animals used for Scientific Purposes. *Official Journal of the European Union* L 276 2010 Oct 20: 33-79.
5. Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to medicinal products for human use. *Official Journal of the European Union* L 311, 2001 Nov 28.
6. Directive 2001/82/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to veterinary medicinal products. *Official Journal of the European Union* L 311, 2001 Nov 28.
7. Schutte K, Szczepanska A, Halder M *et al.* Modern science for better quality control of medicinal products, Towards global harmonization of 3Rs in biologicals: The report of an EPAA workshop. *Biologicals* 2017 (48): 55-65.
8. Hartung T, Aaberge I, Berthold S *et al.* Novel pyrogen tests based on the human fever reaction. The report and recommendations of ECVAM Workshop 43. *ATLA*; 2001 (29): 99-123.
9. Vaccines for veterinary use (Supplement 7.7) – Harmonisation with VICH Guidelines 41 and 44 and deletion of the TABST, adopted at the 142nd Session of the European Pharmacopoeia Commission; Pharmeuropa; Useful information; September 2012 (revised February 2013) [available from: http://pharmeuropa.edqm.eu/home/menupage/English/Useful%20Information/Vaccinesforveterinaryuse_E.pdf, accessed 2018, Feb 12].
10. Technical Guide for elaboration and use of monographs for vaccines and Immunological Veterinary Products [available from: https://www.edqm.eu/sites/default/files/technicalguide-monographs-vaccines-immunological-veterinary-medicinal-products-2016.pdf, accessed 2018, Feb 12].
11. Krämer B, Bruckner L, Daas A *et al.* Collaborative study for validation of a serological potency assay for rabies vaccine (inactivated) for veterinary use. Pharmeur Bio Sci Notes 2010 (2): 37-55.
12. Xing D, Maes A, Behr-Gross ME *et al.* Collaborative study for the standardisation of the histamine sensitizing test in mice and the CHO cell-based assay for the residual toxicity testing of acellular pertussis vaccines. *Pharmeur Bio Sci Notes* 2010 (1): 51-64.
13. Isbrucker R, Daas A, Wagner L *et al.* Transferability study of CHO cell clustering assays for monitoring of pertussis toxin activity in acellular pertussis vaccines, *Pharmeur Bio Sci Notes* 2016: 97-114.
14. Morgeaux S, Variot P, Daas A *et al.* Validation of a new ELISA method for *in vitro* potency testing of hepatitis A vaccines, *Pharmeur Bio Sci Notes* 2013: 64-92.
15. Winsnes R, Sesardic D, Daas A *et al.* Collaborative study on a guinea pig serological method for the Assay of Acellular Pertussis vaccines, *Pharmeur Bio* 2009 (1): 19-32.
16. Winsnes R, Sesardic D, Daas A *et al.* Collaborative Study for the Validation of Serological Methods for Potency Testing of Diphtheria Toxoid Vaccines – Part 2, *Pharmeur Bio* 2006 (1): 73-88.

17. Winsnes R, Hendriksen C. Collaborative Study for the Validation of Serological methods for Potency Testing of Tetanus Toxoid Vaccines for Human Use – Summary of all three phases, *Pharmeur Special Issue Bio* 2001 (2): 75-78.

18. Guideline on plasma-derived medicinal products [guideline]. Ref: EMA/CHMP/BWP/706271/2010; EMA; 2011 July.

19. Guideline on the replacement of rabbit pyrogen testing by an alternative test for plasma derived medicinal products [guideline]. Ref: EMEA/CHMP/BWP/452081/2007; EMEA; 2009 Apr.

20. Charton E. Annual report of activities. Reduction and replacement of animal tests in the European Pharmacopoeia: recent developments for monographs on biological substances *Pharmeuropa* 1999; 11 (2): 197-199.

21. Charton E. Hormones: the role of the European Pharmacopoeia. In: Alternatives to Animals in the Development and Control of Biological Products for Human and Veterinary Use. *Dev Biol. Stand.* 1999; 101: 159-167.

22. Briançon F, Daas E, De Kaste D, *et al.* Replacement of the bioassay by a liquid chromatographic assay in the calcitonin (salmon) monograph. Pharmeuropa 1999; 11 (2): 306-312.

23. Charton E, Castle P. Reduction, replacement and refinement of animal tests in the European Pharmacopoeia: recent developments for monographs on biological substances and preparations. *Pharmeuropa* 2001; 13 (1): 94-96.

24. Bristow A and Charton E. Progress and problems in the replacement of *in vivo* pharmacopoeial bioassays for hormones. In: Advancing science and elimination of the use of laboratory animals for development and control of vaccines and hormones. *Dev. Biol.* (2002) 111: 79-88.

25. Charton E. European Pharmacopoeia activities – an overview. Proceedings of the International Conference Alternatives to Animal testing: New approaches in the Development and Control of Biologicals: Dubrovnik, Croatia; 2008 April 23-24. Council of Europe 2008: 11-16.

26. Guideline on the principles of regulatory acceptance of 3Rs (replacement, reduction, refinement) testing approaches [guideline]. Ref: EMA/CHMP/CVMP/JEG-3Rs/450091/2012; EMA 2016 Dec.

27. Bristow A, Charton E. Assessment of the suitability of a capillary zone electrophoresis method for determining isoform distribution of erythropoietin. *Pharmeuropa* 1999; 11 (2): 290-300.

28. Erythropoietin concentrated solution, monograph 1316. *Pharmeuropa* 2002; 14 (1).

29. Burns C, Bristow AF, Daas A, *et al.* Collaborative study for the establishment of erythropoietin BRP batch 4. *Pharmeur Bio Sci Notes* 2015: 246-253.

30. Burns C, Bristow AF, Buchheit KH, *et al.* Establishment of Erythropoietin for physicochemical tests CRS batch 1. *Pharmeur Bio Sci Notes* 2015: 99-117.

31. Storring PL, Gaines Das RE, Mulders JW, *et al.* Physicochemical methods for predicting the biological potency of recombinant follicle stimulating hormone: an international collaborative study of isoelectric focusing and capillary zone electrophoresis. *Biologicals* 2002: 30 (3): 217-34.

32. Halder M, Depraetere F, Delannois A, *et al.* Recommendations of the VAC2VAC workshop on the design of multi-centre validation studies. *Biologicals* [in press].

33. Recommendation to marketing authorisation holders, highlighting the need to ensure compliance with 3Rs methods described in the European Pharmacopoeia. Applicable to all medicinal products regardless of type. Ref: EMA/CHMP/CVMP/JEG-3Rs/2521372012. EMA; 2012 July.

34. Gross S, Janssen SWJ, de Vries B, *et al.* Collaborative study for the validation of alternative *in vitro* potency assays for human tetanus immunoglobulin. *Pharmeur Bio Sci Notes* 2009 (1): 11-26.

35. Gross S, Janssen SWJ, de Vries B, *et al.* Collaborative study for the validation of alternative *in vitro* potency assays for human tetanus immunoglobulins. *Biologicals* 2010: 38: 501-510.

36. Recommendation to marketing authorisation holders, highlighting recent updates for reduction, refinement and replacement (3Rs) methods described the European Pharmacopoeia. Applicable to human vaccines against hepatitis A. Ref: EMA/CHMP/CVMP/JEG-3Rs/243112/2015. EMA; 2015 May.

37. Recommendation to marketing authorisation holders, highlighting recent measures in the human field to promote replacement, reduction, and refinement (3Rs) measures described in the European Pharmacopoeia. Applicable to human vaccines from 01/01/2018. Ref: EMACHMP/CVMP/3Rs/614768/2017-corr. EMA; 2017 Dec.

38. Recommendation to marketing authorisation holders, highlighting recent measures in the veterinary field to promote replacement, reduction, and refinement (3Rs) measures described in the European Pharmacopoeia. Ref: EMA/CHMP/CVMP/3Rs/614768/2017. EMA; 2017 Dec.

39. Recommendation to marketing authorisation holders for veterinary vaccines, highlighting the need to update marketing authorisations to remove the target animal batch safety test (TABST) following removal of the requirement from the European Pharmacopoeia. Applicable to veterinary vaccines. Ref: EMA/CHMP/CVMP/JEG-3Rs/746429/2012. EMA; 2013 May.

40. Recommendation to marketing authorisation holders, highlighting recent measures in the veterinary field to promote reduction, refinement and replacement (3Rs) measures described in the European Pharmacopoeia. Applicable to veterinary vaccines from 01/01/2017. Ref: EMA/CVMP/3Rs/336802/2017. EMA; 2017 Dec.

Appendix

Table 1 – *Vaccines for veterinary use – 3Rs activity 2007-2017*

Ph. Eur. texts	Revisions
Vaccines for veterinary use (0062)	R1: general deletion of the target animal batch safety test R1: promotion of consistency of production R1: no test for specified extraneous agents needed any more for inactivated vaccines produced using healthy flocks since introduction of a reference to chapter *5.2.13* on Healthy flocks R2: development safety test performed with 8 animals per group instead of 10/8 birds older than 3 weeks or 10 birds younger than 3 weeks per group instead of 20 (*5.2.6*) R2: for inactivated vaccines, identification by antibody induction in animals replaced by any suitable method, e.g. NAT or combined with the batch potency test (using the same animals for both tests)
Anthrax spore live vaccine for vet. use (0441) Avian infectious bronchitis vaccine (live), freeze-dried (0442) Marek's disease vaccine (live) (0589) Rabies vaccine (live, oral) for foxes and raccoon dogs (0746)	R1: deletion of the batch safety test
Salmonella Enteritidis vaccine (live, oral) for chickens (2520) Salmonella Typhimurium vaccine (live, oral) for chickens (2521) Turkey infectious rhinotracheitis vaccine (live) (2461) *Bordetella bronchiseptica* vaccine (live) for dogs (2525)	R1: no batch safety test required
Aujeszky's disease vaccine for pigs (inactivated) (0744) Bovine viral diarrhoea vaccine (inactivated) (1952) Calf coronavirus diarrhoea vaccine (inactivated) (1953) Calf rotavirus diarrhoea vaccine (inactivated) (1954) Canine adenovirus vaccine (inactivated) (1298) Canine parvovirosis vaccine (inactivated) (0795) Equine herpesvirus vaccine (inactivated) (1613) Feline calicivirosis vaccine (inactivated) (1101) Feline infectious enteritis (feline panleucopenia) vaccine (inactivated) (0794) Feline viral rhinotracheitis vaccine (inactivated) (1207) Mycoplasma gallisepticum vaccine (inactivated) (1942) Porcine enzootic pneumonia vaccine (inactivated) (2448) Porcine parvovirosis vaccine (inactivated) (0965)	R1: deletion of the batch safety test R2: development safety test performed with 8 pigs per group instead of 10. R2: identification by antibody induction in animals replaced by any suitable method
Rabbit haemorrhagic disease vaccine (inactivated) (2325)	R1: deletion of the batch safety test R2: development safety test performed with 8 pigs per group instead of 10 R2 conditions for omission of the 2nd inactivation test included R2: identification by antibody induction in animals replaced by any suitable method

R1 = replacement of a test by an *in vitro* test or removal of test.
R2 = reduction in the number of animals required.
R3 = refinement of test to cause less distress, for example by use of more humane end-points.

Ph. Eur. texts	Revisions
Rabies vaccine (inactivated) for veterinary use (0451)	R1: deletion of the batch safety test R2: development safety test performed with 8 pigs per group instead of 10 R2: identification by antibody induction in animals replaced by any suitable method R2: Batch potency test by a serological assay (following BSP105 collaborative study) R3: Possibility to replace the lethal end-point by more humane end-points in the potency assay
Aujeszky's disease vaccine (live) for pigs for parenteral administration, freeze-dried (0745)	R1: deletion of the batch safety test R1: antibody induction test replaced by an *in vitro* method to identify the vaccine (infection of susceptible cell cultures instead of animals)
Avian infectious bronchitis vaccine (inactivated) (0959) Avian infectious bursal disease vaccine (inactivated) (0960) Avian paramyxovirus 3 vaccine (inactivated) for turkeys (1392) Egg drop syndrome '76 vaccine (inactivated) (1202) Equine influenza vaccine (inactivated) (0249) Feline chlamydiosis vaccine (inactivated) (2324) Newcastle disease vaccine (inactivated) (0870) Porcine influenza vaccine (inactivated) (0963)	R1: deletion of the batch safety test R1: deletion of the test for specified extraneous agents following introduction of a reference to chapter *5.2.13* on Healthy flocks R2: development safety test performed with 8 birds per group instead of 20 R2: identification by antibody induction in animals replaced by any suitable method
Avian infectious bursal disease (Gumboro disease) vaccine (live), freeze-dried (0587) Fowl-pox live vaccine, freeze-dried (0649) Newcastle disease vaccine (live), freeze-dried (0450)	R1: deletion of the batch safety test R2: development safety test performed with 8 birds older than 3 weeks or 10 birds younger than 3 weeks per group instead of 20
Avian infectious laryngotracheitis vaccine (live), for chickens (1068) Avian viral tenosynovitis vaccine (live) (1956)	R1: deletion of the batch safety test R2: development safety test performed with 8 chickens older than 3 weeks or 10 chickens younger than 3 weeks per group instead of 20
Duck plague vaccine (live) (1938) Duck viral hepatitis type I vaccine (live) (1315)	R1: deletion of the batch safety test R2: development safety test performed with 8 ducks older than 3 weeks or 10 ducks younger than 3 weeks per group instead of 20
Infectious chicken anaemia vaccine (live) (2038)	R1: deletion of the batch safety test R2: development safety test performed with 8 chickens per group instead of 20 R3: housing of laying hens and young chickens in stable groups of compatible individuals rather than individually
Avian infectious encephalomyelitis vaccine (live) (0588)	R1: deletion of the batch safety test R2: development safety test performed with 8 chickens per group instead of 20
Coccidiosis vaccine (live) for chickens (2326)	R1: deletion of the batch safety test R2: development safety test performed with 10 chickens instead of 20
Bovine leptospirosis vaccine (inactivated) (1939)	R1: deletion of the batch safety test R2: development safety test performed with 8 cattle per group instead of 10 R1: introduction of an *in vitro* batch potency test R1: promotion of consistency of production

R1 = replacement of a test by an *in vitro* test or removal of test.
R2 = reduction in the number of animals required.
R3 = refinement of test to cause less distress, for example by use of more humane end-points.

Ph. Eur. texts	Revisions
Canine leptospirosis vaccine (inactivated) (0447)	R1: introduction of an *in vitro* batch potency test for use with non-adjuvanted vaccines after validation, and extended to all vaccines R1: promotion of consistency of production R1: deletion of the batch safety test R2: development safety test performed with 8 dogs per group instead of 10
Bovine parainfluenza virus vaccine (live), freeze-dried (1176) Bovine respiratory syncytial virus vaccine (live), freeze-dried (1177) Canine distemper vaccine (live), freeze-dried (0448) Canine parainfluenza virus vaccine (live) (1955) Canine parvovirosis vaccine (live) (0964) Distemper vaccine (live) for mustelids, freeze-dried (0449) Feline infectious enteritis (feline panleucopenia) vaccine (live) (0251) Infectious bovine rhinotracheitis vaccine (live), freeze-dried (0696)	R1: deletion of the batch safety test R2: development safety test performed with 5 animals per group (not increased to 8)
Brucellosis vaccine (live) (*Brucella melitensis* Rev. 1 strain), freeze-dried, for veterinary use (0793)	R1: deletion of the batch safety test R2: 'Fifty per cent persistence time' performed on each batch of vaccine using 32 mice replaced by a test for 'residual virulence' performed on the master seed lot and on one representative batch of vaccine. R3: immunogenicity test in sheep replaced by a test in mice
Clostridium botulinum vaccine for veterinary use (0360) *Clostridium chauvoei* vaccine for veterinary use (0361) Feline calicivirosis vaccine (live), freeze-dried (1102) Feline viral rhinotracheitis vaccine (live), freeze-dried (1206) Myxomatosis vaccine (live) for rabbits (1943)	R1: deletion of the batch safety test R2: development safety test performed with 8 animals per group instead of 10
Clostridium novyi (type B) vaccine for veterinary use (0362)	R1: deletion of the batch safety test R2: development safety test performed with 8 animals per group instead of 10 R3: introduction of a serological evaluation of the batch potency test
Clostridium perfringens vaccine for veterinary use (0363) *Clostridium septicum* vaccine for veterinary use (0364)	R1: deletion of the batch safety test R2: waiver for the test for residual toxicity test on the final product by the manufacturer R2: development safety test performed with 8 animals per group instead of 10
Colibacillosis inactivated vaccine, neonatal ruminant (0961) Colibacillosis inactivated vaccine, neonatal piglet (0962)	R1: deletion of the batch safety test R2: development safety test performed with 8 pregnant animals per group instead of 10
Swine-fever vaccine (live) classical, freeze-dried (0065)	R1: deletion of the batch safety test R2: development safety test performed with 8 piglets/ pregnant sows per group instead of 10

R1 = replacement of a test by an *in vitro* test or removal of test.
R2 = reduction in the number of animals required.
R3 = refinement of test to cause less distress, for example by use of more humane end-points.

Ph. Eur. texts	Revisions
Feline leukaemia vaccine (inactivated) (1321)	R1: deletion of the batch safety test R2: identification by antibody induction in animals replaced by any suitable method
Foot-and-mouth disease (ruminants) vaccine (inactivated) (0063)	R1: deletion of the batch safety test R2: development safety test performed with 8 cattle per group instead of 10 R2: identification by antibody induction in animals replaced by any suitable method
Fowl cholera vaccine (inactivated) (1945)	R1: deletion of the batch safety test R2: development safety test performed with 8 birds older than 3 weeks or 10 birds younger than 3 weeks per group instead of 20. R2: identification by antibody induction in animals replaced by any suitable method
Infectious bovine rhinotracheitis vaccine (inactivated) (2674)	R1: promotion of consistency of production R1: introduction of an *in vitro* batch potency test
Mannheimia vaccine (inactivated) for cattle (1944) Mannheimia vaccine (inactivated) for sheep (1946) Neonatal piglet colibacillosis vaccine (inactivated) (0962) Neonatal ruminant colibacillosis vaccine (inactivated) (0961) Pasteurella vaccine (inactivated) for sheep (2072)	R1: deletion of the batch safety test R2: development safety test performed with 8 animals per group instead of 10 R2: identification by antibody induction in animals replaced by any suitable method
Porcine actinobacillosis vaccine (inactivated) (1360) Porcine progressive atrophic rhinitis vaccine (inactivated) (1361)	Exception: batch safety test replaced by test for residual toxicity. R2: development safety test performed with 8 animals per group instead of 10
Tetanus vaccine for veterinary use (0697)	Exception: batch safety test replaced by test for residual toxicity. R2: development safety test performed with 8 animals per group instead of 15 R3: introduction of a serological evaluation for the potency test
Salmonella Enteritidis vaccine (inactivated) for chickens (1947) Salmonella Typhimurium vaccine (inactivated) for chickens (2361)	R1: deletion of the batch safety test R2: development safety test performed with 8 chickens older than 3 weeks per group instead of 10 R2: identification by antibody induction in animals replaced by any suitable method
Swine erysipelas vaccine (inactivated) (0064)	R1: deletion of the batch safety test R2: development safety test performed with 8 pigs per group instead of 10 R3: introduction of a serological evaluation for the batch potency test R2: identification by antibody induction in animals replaced by any suitable method
Fish vaccines	
Furunculosis vaccine (inactivated, oil-adjuvanted, injectable) for salmonids (1521)	R1: deletion of the batch safety test R2: reduction from 200 to 60 fish to be used for Immunogenicity R2: identification by antibody induction in animals replaced by any suitable method

R1 = replacement of a test by an *in vitro* test or removal of test.
R2 = reduction in the number of animals required.
R3 = refinement of test to cause less distress, for example by use of more humane end-points.

Ph. Eur. texts	Revisions
Vibriosis (cold-water) vaccine (inactivated) for salmonids (1580)	R1: deletion of the batch safety test R2: reduction from 200 to 60 fish to be used for Immunogenicity R2: identification by antibody induction in animals replaced by any suitable method
Vibriosis vaccine (inactivated) for salmonids (1581)	R1: deletion of the batch safety test R2: reduction from 200 to 60 fish to be used for Immunogenicity R2: identification by antibody induction in animals replaced by any suitable method
Yersiniosis vaccine (inactivated) for salmonids (1950)	R1: deletion of the batch safety test R2: reduction from 120 to 60 fish to be used for Immunogenicity R2: identification by antibody induction in animals replaced by any suitable method

R1 = replacement of a test by an *in vitro* test or removal of test.
R2 = reduction in the number of animals required.
R3 = refinement of test to cause less distress, for example by use of more humane end-points.

Table 2 – *Vaccines for human use – 3Rs activity 2007-2017*

Ph. Eur. texts	Revisions
Tests for extraneous agents in viral vaccines for human use (2.6.16) Cell substrates for the production of vaccines for human use (5.2.3)	R1: deletion of the tests in adult mice and guinea pigs. R2: testing strategy for extraneous agents to be established based on a risk assessment. Tests in suckling mice and control eggs to be used only if a risk assessment indicates that the tests provide risk mitigation. R2: allow use of molecular methods for specific or broad detection of viruses
Assay of diphtheria vaccine (adsorbed) (2.7.6)	R3 & R2: introduction of a serology assay as an alternative to challenge, with the possibility to use the same animals for the serological assay of the tetanus vaccine
Assay of tetanus vaccine (adsorbed) (2.7.8)	R2: possibility to use the same animals for the serological assay of the diphtheria vaccine
Assay of pertussis vaccine (acellular) (2.7.16)	R2: possibility to use the same animals for the serological assay of the diphtheria and tetanus vaccines
Diphtheria, tetanus and hepatitis B (rDNA) vaccine (adsorbed) (2062)	R2: replacement of the test for specific toxicity of the diphtheria and tetanus components by a requirement to validate the process so that the product if tested would comply with the test R1: deletion of the abnormal toxicity test
Diphtheria, tetanus and pertussis (acellular, component) vaccine (adsorbed) (1931)	R2: replacement of the test for specific toxicity of the diphtheria and tetanus components by a requirement to validate the process so that the product if tested would comply with the test
Diphtheria, tetanus, pertussis (acellular, component) and haemophilus type b conjugate vaccine (adsorbed) (1932)	R2: replacement of the test for specific toxicity of the diphtheria and tetanus components by a requirement to validate the process so that the product if tested would comply with the test R1: replacement of the rabbit pyrogen test by the bacterial endotoxin test R1: deletion of the requirement to resort to animal models each time the manufacturing process is changed R1: deletion of the abnormal toxicity test

R1 = replacement of a test by an *in vitro* test or removal of test.
R2 = reduction in the number of animals required.
R3 = refinement of test to cause less distress, for example by use of more humane end-points.

Ph. Eur. texts	Revisions
Diphtheria, tetanus, pertussis (acellular, component) and hepatitis B (rDNA) vaccine (adsorbed) (1933)	R2: replacement of the test for specific toxicity of the diphtheria and tetanus components by a requirement to validate the process so that the product if tested would comply with the test
In vivo assay of poliomyelitis vaccine (inactivated) (2.7.20)	R2: possibility to waive the *in vivo* assay once it has been demonstrated that the D-antigen determination yields the same result. Introduction of guidance on the implementation of D-antigen testing
Diphtheria, tetanus, pertussis (acellular, component) and poliomyelitis (inactivated) vaccine (adsorbed) (1934)	R2: replacement of the test for specific toxicity of the diphtheria and tetanus components by a requirement to validate the process so that the product if tested would comply with the test R1: deletion of the abnormal toxicity test R2: possibility to waive the *in vivo* assay for the poliomyelitis component once it has been demonstrated that the D-antigen determination yields the same result
Diphtheria, tetanus, pertussis (acellular, component), hepatitis B (rDNA), poliomyelitis (inactivated), and haemophilus type b vaccine (adsorbed) (2067)	R2: possibility to waive the *in vivo* assay for the poliomyelitis component once it has been demonstrated that the D-antigen determination yields the same result R1: deletion of the requirement to resort to animal models each time the manufacturing process is changed
Diphtheria, tetanus, pertussis (acellular, component), poliomyelitis (inactivated) and haemophilus type b conjugate vaccine (adsorbed) (2065)	R2: replacement of the test for specific toxicity of the diphtheria and tetanus components by a requirement to validate the process so that the product if tested would comply with the test R1: deletion of the abnormal toxicity test R2: possibility to waive the *in vivo* assay for the poliomyelitis component once it has been demonstrated that the D-antigen determination yields the same result R1: replacement of the rabbit pyrogen test by the bacterial endotoxin test R1: deletion of the requirement to resort to animal models each time the manufacturing process is changed
Diphtheria, tetanus, pertussis (whole cell) and poliomyelitis (inactivated) vaccine (adsorbed) (2061)	R2: possibility to waive the *in vivo* assay for the poliomyelitis component once it has been demonstrated that the D-antigen determination yields the same result R1: deletion of the requirement to resort to animal models each time the manufacturing process is changed
Diphtheria, tetanus, pertussis (whole cell), poliomyelitis (inactivated) and haemophilus type b conjugate vaccine (adsorbed) (2066)	R2: replacement of the test for specific toxicity of the diphtheria and tetanus components by a requirement to validate the process so that the product if tested would comply with the test R1: deletion of the abnormal toxicity test R2: possibility to waive the *in vivo* assay for the poliomyelitis component once it has been demonstrated that the D-antigen determination yields the same result R1: replacement of the rabbit pyrogen test by the bacterial endotoxin test R1: deletion of the requirement to resort to animal models each time the manufacturing process is changed
Haemophilus type b conjugate vaccine (1219)	R1: deletion of the requirement to resort to animal models each time the manufacturing process is changed R1: deletion of the abnormal toxicity test
Poliomyelitis vaccine (inactivated) (0214)	R2: possibility to waive the *in vivo* assay once it has been demonstrated that the D-antigen determination yields the same result R1: deletion of the abnormal toxicity test

R1 = replacement of a test by an *in vitro* test or removal of test.
R2 = reduction in the number of animals required.
R3 = refinement of test to cause less distress, for example by use of more humane end-points.

Ph. Eur. texts	Revisions
Poliomyelitis vaccine (oral) (0215)	R2: introduction of genome analysis (MAPREC) for screening prior to neurovirulence testing in animals R3: allow the use of transgenic mice to replace monkeys in the neurovirulence assay (for seed lots)
Anthrax vaccine for human use (adsorbed, prepared from culture filtrates) (2188)	R1: deletion of the abnormal toxicity test
Assay of hepatitis A vaccine (2.7.14)	R1: introduction of a validated *in vitro* assay as an alternative to the assay in mice
Hepatitis A (inactivated, adsorbed) and typhoid polysaccharide vaccine (2597)	R1: introduction of a validated *in vitro* assay for hepatitis A potency determination.
Hepatitis A (inactivated) and hepatitis B (rDNA) vaccine (adsorbed) (1526) Hepatitis A vaccine (inactivated, adsorbed) (1107) Hepatitis A vaccine (inactivated, virosome) (1935)	R1: deletion of the abnormal toxicity test
Hepatitis B vaccine (rDNA) (1056)	R1: deletion of the abnormal toxicity test
Human papillomavirus vaccine (rDNA) (2441)	R1: deletion of the abnormal toxicity test
Influenza vaccine (split virion, inactivated) (0158) Influenza vaccine (surface antigen, inactivated) (0869) Influenza vaccine (surface antigen, inactivated, prepared in cell cultures) (2149) Influenza vaccine (surface antigen, inactivated, virosome) (2053) Influenza vaccine (whole virion, inactivated) (0159) Influenza vaccine (whole virion, inactivated, prepared in cell cultures) (2308)	R1: deletion of the abnormal toxicity test
Measles, mumps and rubella vaccine (live) (1057) Measles, mumps, rubella and varicella vaccine (live) (2442)	R1: deletion of the abnormal toxicity test
Measles vaccine (live) (0213) Mumps vaccine (live) (0538) Rubella vaccine (live) (0162) Varicella vaccine (live) (0648)	R2: replacement of the neurovirulence test on seed lots by a requirement to study the neurovirulence during development R1: deletion of the abnormal toxicity test
Meningococcal group C conjugate vaccine (2112) Meningococcal polysaccharide vaccine (0250) Pneumococcal polysaccharide conjugate vaccine (adsorbed) (2150) Pneumococcal polysaccharide vaccine (0966)	R1: deletion of the abnormal toxicity test
Rabies vaccine for human use prepared in cell cultures (0216)	R3: possibility to replace the lethal end-point by more humane end-points in the potency assay R1: promotion of the use of a serology or immunochemical method as an alternative to the assay in mice R1: deletion of the abnormal toxicity test
Shingles (herpes zoster) vaccine (live) (2418)	R1: deletion of the abnormal toxicity test
Tick-borne encephalitis vaccine (inactivated) (1375)	R3: possibility to replace the lethal end-point by more humane end-points in the potency assay R1: deletion of the abnormal toxicity test
Typhoid polysaccharide vaccine (1160) Typhoid vaccine (0156)	R1: deletion of the abnormal toxicity test
Yellow fever vaccine (live) (0537)	R1: deletion of the potency assay in mice R1: deletion of the abnormal toxicity test

R1 = replacement of a test by an *in vitro* test or removal of test.
R2 = reduction in the number of animals required.
R3 = refinement of test to cause less distress, for example by use of more humane end-points.

Table 3 – *Blood products – 3Rs activity 2007-2017*

Ph. Eur. texts	Revisions
Human albumin solution (0255)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*
Human antithrombin III concentrate (0878)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*
Human C1-esterase inhibitor (2818)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*
Human coagulation factor VII (1224)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*
Human coagulation factor VIII (0275)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*
Human coagulation factor IX (1223)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*
Human coagulation factor XI (1644)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*
Human fibrinogen (0024)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*
Human normal immunoglobulin for intramuscular administration (0338)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*
Human normal immunoglobulin for intravenous administration (0918)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*
Human normal immunoglobulin for subcutaneous administration (2788)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*
Human plasma (pooled and treated for virus inactivation) (1646)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*
Human alpha-1-proteinase inhibitor (2387)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*
Human prothrombin complex (0554)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*
Human von Willebrand factor (2298)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*

R1 = replacement of a test by an *in vitro* test or removal of test.
R2 = reduction in the number of animals required.
R3 = refinement of test to cause less distress, for example by use of more humane end-points.

Table 4 – *Biological and biotechnological products – 3Rs activity 2007-2017*

Ph. Eur. texts	Revisions
Tetracosactide (0644)	R1: replacement of animal assay by liquid chromatography
Erythropoietin concentrated solution (1316)	R2: separate standard introduced to minimise the use of Erythropoietin BRP calibrated in International Units
Aprotinin (0580)	R1: deletion of abnormal toxicity test
Aprotinin concentrated solution (0579)	R1: deletion of abnormal toxicity test
Protamine sulfate (0569)	R1: deletion of abnormal toxicity test
Streptokinase concentrated solution (0356)	R1: deletion of abnormal toxicity test
Urokinase (0695)	R1: deletion of pyrogen test (ongoing)

R1 = replacement of a test by an *in vitro* test or removal of test.
R2 = reduction in the number of animals required.
R3 = refinement of test to cause less distress, for example by use of more humane end-points.

Table 5 – *Radiopharmaceuticals – 3Rs activity 2007-2017*

Ph. Eur. texts	Revisions
Technetium (^{99m}Tc) etifenin injection (0585)	R1: replacement of the physiological distribution test by a series of physico-chemical tests.
Technetium (^{99m}Tc) medronate injection (0641)	R1: replacement of the physiological distribution test by a series of physico-chemical tests.

R1 = replacement of a test by an *in vitro* test or removal of test.
R2 = reduction in the number of animals required.
R3 = refinement of test to cause less distress, for example by use of more humane end-points.

Table 6 – *Antibiotics and antimycotics – 3Rs activity 2007-2017*

Ph. Eur. texts	Revisions
Streptomycin sulfate (0053)	R1: deletion of abnormal toxicity test
Dihydrostreptomycin sulfate for veterinary use (0485)	R1: deletion of abnormal toxicity test
Kanamycin acid sulfate (0032)	R1: deletion of abnormal toxicity test
Kanamycin monosulfate (0033)	R1: deletion of abnormal toxicity test
Rifamycin sodium (0432)	R1: deletion of abnormal toxicity test
Griseofulvin (0182)	R1: deletion of abnormal toxicity test
Nystatin (0517)	R1: deletion of abnormal toxicity test

R1 = replacement of a test by an *in vitro* test or removal of test.
R2 = reduction in the number of animals required.
R3 = refinement of test to cause less distress, for example by use of more humane end-points.

Table 7 – *Other products – 3Rs activity 2007-2017*

Ph. Eur. texts	Revisions
Allergen products (1063)	R1: deletion of abnormal toxicity test
Plastic containers (3.1.1.1, 3.1.1.2, 3.1.13, 3.1.14, 3.2.3, 3.2.4, 3.2.5)	R1: deletion of abnormal toxicity test

R1 = replacement of a test by an *in vitro* test or removal of test.
R2 = reduction in the number of animals required.
R3 = refinement of test to cause less distress, for example by use of more humane end-points.

Collaborative study for the establishment of human immunoglobulin BRP replacement batches

D. Karra[1], E. Regourd[2], A. Costanzo[2]

ABSTRACT

Human immunoglobulin products are used for the treatment of a number of diseases, such as primary or secondary immunodeficiencies and autoimmune conditions due to the complete absence of antibodies or the production of defective immunoglobulins. Quality control of human immunoglobulin products is essential to ensure therapeutic functionality and safety. This includes testing for Fc function and anticomplementary activity (ACA), as well as verification of appropriate molecular size distribution using size-exclusion chromatography as prescribed in the European Pharmacopoeia (Ph. Eur.) monographs 0338, 0918, 2788 and 1928. To this end, specific biological reference preparations (BRPs) must be used. Stocks of these BRPs were running low and therefore a collaborative study was run by the European Directorate for the Quality of Medicines & HealthCare (EDQM), under the aegis of the Biological Standardisation Programme, to calibrate replacement batches. Seventeen laboratories, including manufacturers and Official Medicines Control Laboratories, took part in the study. Several batches of candidate BRPs were calibrated against human immunoglobulin (ACA and molecular size) BRP batch 1 and human immunoglobulin (Fc function and molecular size) BRP batch 1 to ensure continuity. Based on the study results, the candidate BRPs were adopted by the Ph. Eur. Commission as Ph. Eur. human immunoglobulin for anticomplementary activity BRP batch 1 and batch 2, Ph. Eur. human immunoglobulin for Fc function BRP batch 1 and batch 2 and Ph. Eur. human immunoglobulin (molecular size) BRP batch 2 and batch 3.

KEYWORDS

Human immunoglobulin, biological reference preparation, European Pharmacopoeia, anticomplementary activity, Fc function, molecular size distribution, collaborative study.

1. INTRODUCTION AND AIM

Immunoglobulin products are listed as ‘essential medicines’ by the World Health Organization (WHO) and are among the most widely used therapeutics globally; therefore, they are in high demand. They can be applied intravenously, intramuscularly or subcutaneously. As highly active molecules, and although their use is considered safe in general, they can lead to serious adverse effects such as anaphylactic reactions or thrombosis; therefore, high quality and stringent safety profiles are required.

The production process of human normal immunoglobulins from large pools of plasma consists of several fractionation, precipitation and purification steps which may lead to modifications of the immunoglobulin molecules, such as aggregation, which may alter their activity or pharmacokinetic properties after infusion to patients. Depending on the origin and on the aggregation process, the resulting aggregates may or may not be detrimental to the quality of

1 D. Karra. Paul-Ehrlich Institut, Paul-Ehrlich-Strasse 51-59, D-63225 Langen, Germany.
2 E. Regourd, A. Costanzo (corresponding author’s e-mail: angele.costanzo@edqm.eu), European Directorate for the Quality of Medicines & HealthCare, Department of Biological Standardisation, OMCL Network & HealthCare (DBO), Council of Europe, Strasbourg, France.

the immunoglobulin product [1], and therefore determination of the molecular size distribution alone is not sufficient. For this reason, the European Pharmacopoeia (Ph. Eur.) monographs *Human normal immunoglobulin for intramuscular administration (0338)* [2], *Human normal immunoglobulin for intravenous administration (0918)* [3], *Human normal immunoglobulin for subcutaneous administration (2788)* [4] and *Anti-T lymphocyte immunoglobulin for human use, animal (1928)* [5] require that the anticomplementary activity (ACA), the Fc function and/or the molecular size distribution of human normal immunoglobulin therapeutic products be controlled and define limits for these tests – hence the need for specific reference standards to be used for these determinations.

The current Ph. Eur. biological reference preparations (BRPs) for human immunoglobulin, i.e. the (ACA and molecular size) BRP and the (Fc function and molecular size) BRP, were established in 2010 following a collaborative study (BSP099) [6] run by the European Directorate for the Quality of Medicines & HealthCare (EDQM) under the aegis of the Biological Standardisation Programme (BSP). They are used as working references in the tests for ACA and Fc function, as well as molecular size determination by size-exclusion chromatography (SEC) in accordance with the specifications of the Ph. Eur. monographs listed above. Stocks of these reference preparations were running low and needed to be replaced. In addition, the human immunoglobulin (molecular size) BRP, specifically dedicated to determination of the distribution of molecular size, was exhausted. Therefore, a new project (BSP122) was initiated by the EDQM with the aim of establishing replacement batches.

In the past, reference preparations for human normal immunoglobulin were established for 2 or more of the required tests. However, this led to the necessity to revise the pharmacopoeial texts when the attributes of a reference preparation differed from the previous one. It was thus decided to establish ‘single-use’ reference preparations to avoid such frequent changes in the future.

The project was run in 2 phases, a preliminary phase (Phase 1) to assess the candidate materials with regard to their suitability for the intended use, followed by an international collaborative study (Phase 2) for their calibration for the various tests mentioned above.

Four batches of candidate materials were procured from European manufacturers of therapeutic immunoglobulin products. Phase 1 was carried out in 2 laboratories to verify whether the candidate materials were appropriate for the intended use. The results from Phase 1 showed that samples C and D were suitable for the tests for ACA, Fc function and molecular size, but it became clear that samples A and B could not be used for the ACA test as no complement consumption was observed. However, they were shown to be suitable for the other 2 tests.

During Phase 2 of the project for calibration of the candidate materials, samples A and B were therefore investigated as potential working standards for Fc function and/or molecular size tests, and samples C and D for the ACA test only. The experimental phase ran from November 2016 to January 2017 and the results are reported herein. Since the material contained in each vial largely exceeds the amount needed to perform one assay, participants also carried out additional assays, 7 and 14 days after reconstitution or first opening of the vials, to assess the stability of the candidate BRPs (cBRPs).

2. PARTICIPANTS

Seventeen participants, including European manufacturers of immunoglobulin products and Official Medicines Control Laboratories (OMCLs), took part in the study. Some of them carried out only a subset of tests. Participating laboratories are listed in alphabetical order by country in section 8. They are referred to herein by a randomly allocated code number which does not necessarily correspond to the order of listing.

3. MATERIALS, METHODS AND STUDY DESIGN

Materials were dispatched on ice by the EDQM and stored by the participants at 2-8 °C upon receipt and until use. Participants received sufficient materials to carry out the necessary number of independent assays depending on the subset of tests that they would perform. They also received additional vials of reference preparations to carry out assays for the stability assessment, i.e. one assay 7 days after reconstitution/opening and one assay 14 days after reconstitution/opening, the day of reconstitution being referred to as Day 1 (see section 3.7).

The study design was detailed in the study protocol which was provided to participating laboratories together with appropriate electronic reporting sheets. For samples A and B, participants were instructed to dilute, just before use, a sufficient volume from each vial 1:2 in *water R* to obtain a 50 mg/mL immunoglobulin solution. However, some laboratories observed unexpected haemolysis during the performance of the Fc function test when following these instructions. The acidic pH of the samples was considered as a potential cause. Participants were thus instructed to use albumin barbital buffer solution for direct dilution of the samples to 30 and 40 mg/mL instead.

3.1. Candidate BRPs

After reconstitution (where necessary) and taking out (with precautions to avoid contaminations) the necessary volume to carry out the assay, vials were stored at 2-8 °C for up to 14 days for the performance of the stability investigation (see section 3.7).

- **Samples A and B:** candidate Ph. Eur. human immunoglobulin (Fc function, molecular size) BRPs. The materials are sterile 10 % liquid formulations of highly purified immunoglobulin G (IgG), pH 4.9. Each unit of plasma used to prepare the final batches was tested by the manufacturer and found to be non-reactive for antibodies to HIV (1 and 2) and HCV, and negative for hepatitis B surface antigen (HBsAg).
- **Samples C and D:** candidate Ph. Eur. human immunoglobulin for anticomplementary activity BRPs. Each vial contains 10 g of lyophilised sterile human normal immunoglobulin. The candidate materials were to be reconstituted just before use with 180.4 mL *water R* per vial to obtain a 50 mg/mL solution. The pH of the reconstituted solution was approximately 6.5.

3.2. Reference preparations: samples E and F

Ph. Eur. human immunoglobulin (ACA and molecular size) BRP batch 1 (sample E, EDQM catalogue number Y0001504) and human immunoglobulin (Fc function and molecular size) BRP batch 2 (sample F, EDQM catalogue number Y0001512) were provided to participants by the EDQM to be used as reference preparations in each assay. For use, the BRPs were to be reconstituted as prescribed in their respective leaflets, appended to the study protocol.

3.3. Assay methods and study design

Participants were asked to submit data from 3 independent valid assays for each of the tests they had agreed to perform, following the design described below. Adjusting the pH of the immunoglobulin samples is permitted by the monographs; however, it was shown during the preliminary phase that this was not necessary for the samples included in the study. Therefore, participants were requested to test the samples without adjusting the pH to 7. Additional reagents, i.e. complement, haemolysin, etc., were to be procured by participants from their usual suppliers or produced in-house, as applicable in their routine procedures. A summary of the reagents used in the various tests is given in Tables 1-3.

A short-term stability investigation was also carried out by some participants. The vials used for assay 1 were stored at 2-8 °C for up to 14 days to carry out the stability assessment (see section 3.7). One laboratory also retested the vials used for assays 2 and 3.

3.4. Test for Fc function

The Fc function test was performed as described in Ph. Eur. chapter 2.7.9, *Test for Fc function of immunoglobulin* [7] for samples A, B and F using a suitable (in-house or commercial) complement preparation. Two vials of each candidate material and 1 vial of BRP (sample F) were to be included in each assay. Each of them was tested in duplicate at 2 concentrations, i.e. 30 mg and 40 mg. Participants were asked to return raw data as well as the Index of Fc function (IFc) calculated relative to the BRP.

Table 1 – *Fc Function test: details of additional reagents used by participants*

Fc function					
Lab	Complement		Rubella antigen		Comments
	Supplier / Lot	Titre (CH_{50}/mL)	Supplier	Titre	
2	In-house (pool of at least 10 guinea pigs) Batch 11189 (09/12/2015)	241,05	Siemens Batch 400126	1:1024	
3	In-house (pool of 15 to 18 guinea pigs)	156	n.r.	n.r.	
8	Harlan batch 15-9459 Charles River batch SC-251	264	Aalto Bio Reagents Batch 3356	n.r.	Complement: a mix 1:1 of Harlan and Charles River complement preparation was used for all assays (in house batch number 16/166 (25/10/2016). Partial hemolysis observed in assay 1
13	In-house (pool of 10 guinea pigs)	160	In-house Batch 443	256 HIT/mL	
14	Cedarlane Batch 4424 (Exp. 30/11/2017)	226 (working conc. 150)	Aalto Batch 3667 (Exp. 28/04/2018)	1.19 mg of rubella antigen/mL	
15	Sigma Batch SLBG4298V	n.r.	Aalto Bio Reagents Batch 2996	80 IU/mL	used a SRH method
17	Charles River Batch SC02.03.16 (030816)	226.5	Aalto Batch 3356 (051015)	Protein conc: 1.10 g/L	method B Ph. Eur.; 10 and 20 g/L

Lab: laboratory; n.r.: not reported.

3.5. Anticomplementary activity (ACA)

The ACA test was performed as described in Ph. Eur. chapter 2.6.17, *Test for anti-complementary activity of immunoglobulin* [8] for samples C, D and E using a suitable (in-house or commercial) complement preparation. For each independent assay, 2 vials of each sample and 1 vial of BRP were reconstituted and tested in duplicate.

All samples served as both negative and positive controls. For the negative control, 0.2 mL of solution was used. During Phase 1, samples C and D were tested at different concentrations and it appeared that using 0.6 mL often led to ACA lower than the current requirement for the positive control. The performance of the ACA test is linked to the quality of the additional reagents used in the test, such as the complement. In order to avoid being at the lower edge of the prescribed limits, which might result in falsely judging the assays as invalid, it was decided to use a larger volume of candidate materials for the test.

Therefore, for the positive control, participants were requested to use 0.8 mL of samples C and D, and 0.6 mL of sample E. For all samples, raw data and estimated ACA were to be returned.

3.6. Distribution of molecular size by size-exclusion chromatography (SEC)

The distribution of molecular size was determined according to the specifications of the above-mentioned monographs for samples A and B with sample E as reference preparation. All laboratories carried out 2 independent assays using 2 vials of each test sample and 1 vial of reference preparation per assay. Each vial was to be injected once. Participants were asked to return copies of the chromatograms and raw data of retention times as well as peak areas for the monomer, dimer and aggregates/polymers. If the monomer and dimer peak areas could not be calculated individually, the sum of monomer and dimer peak areas was to be

reported instead. In addition, the relative retention (RR) of the dimer to the monomer was to be calculated.

Table 2 – *ACA test: details of additional reagents used by participants*

ACA						
Lab	Complement		Haemolysin			Comments
	Supplier / Lot	Titre (CH_{50}/mL)	Supplier / Lot	MHU/mL	Dilution	
2	In-house (pool of at least 10 guinea pigs) assays 1-3: batch 060716 (06/07/2016) assay 4: batch 280916 (28/09/2016)	Assays 1-3: 280 Assay 4: 261	Siemens Batch 302188 (08/10/2016) Amboceptor (ORLC255)	1	1:400	dilution of commercial stock in Gelatin Barbital Buffer Solution
3	In-house (pool of 15 to 18 guinea pigs) Batch 263/16BC (20/07/2016)	Assay 1: 244 Assay 2: 239 Assay 3: 237 Assay day 7: 250 Assay day 14: 237	Siemens Batch 302188 Amboceptor (ORLC25)	1	1:150	
5		270	Siemens Batch 302188	1	1:800	
13	In-house (pool of 10 guinea pigs) Batch 22 (12/04/2016)	160	Virion/Serion # NO9002	1	1:600	
14	Merck Batch 2628218 (Exp. 12/2019)	Assays 1 & 2: 205; assay 3: 173 Assay day 7: 210 Assay day 14: 222 (working conc. 120 for all assays)	Biomerieux Batch 1004415320 (Exp. 23/05/2017)	10	n.r.	
16	Quidel Corp. Batch 083400 (10/2016)	216	Virion/Serion Batch SIF.AT	1000	n.r.	
17	Charles River Batch SC13.09.16 (080916)	206.4	Biomerieux Batch 1004673600	800	n.r.	

Lab: laboratory; n.r.: not reported.

Table 3 – *Molecular size distribution test: information on chromatographic conditions and columns used by participants*

Lab	Concentration (g/L)	Volume injected (µL)	Column	Dilution buffer	Comments
1	10	30	TSK G3000SW; TOSOH Bioscience (7.5 mm x 60 cm)	n.r.	
2	8.334	10	TSK gel G3000SW; TOSOH Bioscience P/N 05103 (7.5 mm x 60 cm)	NaCl 9 g/L	
3a	10	20	TSK gel G3000SW; TOSOH Corporation (7.5 mm x 60 cm)	n.r.	
3b	10	25	TOSOHaas (7.5 mm x 60 cm)	n.r.	
4	50	5	TSK gel G3000 SW; TOSOH Bioscience (7.5 mm x 30 cm; 10 µm)	water	Assays were performed at various concentrations with the stated injection volumes for all samples except assay 1 (29/11/2016): 5 g/L for samples A & B; 10 g/L for sample E
	50	10	TSK G3000 SW; TOSOH Bioscience (7.5 mm x 30 cm; 10 µm)	water	
	10	30	TSK G3000 SW; TOSOH Bioscience (7.5 mm x 30 cm; 10 µm)	water	
5	10	20	TSK 3000SW; TOSOH Bioscience (7.5 mm x 60 cm)	NaCl 9 g/L	
6	50	20	TSK G3000SW (7.5 mm x 60 cm)	n.r.	
7	50 g/L for samples A & B 5 g/L for sample E	20	TSK-GEL® G3000SWXL; Tosoh Bioscience (7.8 mm x 30 cm)	water	
8	50	4	TSKgel G3000 SWXL; TOSOH Bioscience # 08541 (7.8 mm x 30 cm)	n.r.	
9	50	3	TSKgel G3000SW XL; TOSOH Corporation (7.8 mm x 30 cm) + TSKgel SWXL Guardcolumn	n.r.	
10	5	20	TSKgel G3000 SWXL (7.8 mm x 30 cm)	n.r.	
11	50	100	TSKgel G3000 SWXL; TOSOH Bioscience (7.8 mm x 30 cm)	NaCl 9 g/L	
12	10	20	TSK gel G3000SW; TOSOH Bioscience (7.8 mm x 60 cm)	n.r.	
13	10	25	TSKgel G3000SWXL; TOSOH Bioscience (7.8 mm x 30 cm)	n.r.	
14	5	30	Superose 12 Pharmacia (10 mm x 30 cm)	n.r.	
15	10	20	G3000SWXL; TOSOH Bioscience (7.8 mm x 30 cm)	n.r.	
16	50	10	TSKgel BioAssist® G3SWXL; Tosoh (7.8 mm x 30 cm)	n.r.	

Lab: laboratory; n.r.: not reported.

3.7. Stability investigation

Vials from assay 1 (Day 1) for each test, stored at 2-8 °C were retested on Days 7 and 14 after reconstitution or first opening in the ACA, Fc function and SEC tests, as appropriate. Laboratory 13 also tested vials from assays 2 and 3. Participants included the samples and the appropriate reference preparation in each assay; each vial was tested once at each time point. Fresh vials of reference preparation were used in each assay.

4. RESULTS AND DISCUSSION

Seventeen laboratories participated in this study; in this report, they are referred to by their randomly allocated code numbers (1 to 17). Both raw data and calculated results were reported. The data set was analysed centrally at the EDQM and calculations were compared to those submitted by participants to exclude reporting and/or calculation errors. Participants' calculations are reported in the appendix for information.

4.1. Fc function test

Of the 7 laboratories that performed the Fc function investigation, 6 carried out the test according to the Ph. Eur. method. They reported results from at least 3 assays using 2 different vials, as requested in the study protocol. Laboratory 15 carried out a single radial haemolysis (SRH) method which delivers different endpoints. Their results could therefore not be included in the tables and calculations. A summary of the additional reagents used for the assays is given in Table 1. Laboratory 13 used a setup which did not allow testing of 2 vials of each test sample concomitantly. They therefore carried out 2 separate assays, including 2 vials of test sample (A or B) and the reference preparation in each of them. These were considered in the overall combination as independent assays.

The results of central calculations performed at the EDQM on the raw data submitted by the participants are presented in Table 4. Listed are the mean corrected slopes for all samples and the complement control, and the Index of Fc function (IFc) for samples A and B relative to the BRP (sample F). For all assays, the centrally calculated IFc was consistent with the values reported by participants, and all were at or above the limit required by the BRP leaflet, i.e. 60 %. The overall centrally calculated IFc means (and corresponding standard deviations) were 99.1 % (SD = 7.8) and 97.8 % (SD = 11.8) for samples A and B, respectively. Results reported by participants for their own calculations were almost identical: 99.0 % (SD = 7.9) and 97.6 % (SD = 11.9), respectively.

4.2. Anticomplementary activity

Seven laboratories performed the ACA test on Day 1. An overview of the central calculation results based on the raw data submitted by participants is presented in Table 5. The mean results per laboratory were calculated and the mean of means is shown at the bottom of Table 5, together with the SD. The overall calculations were performed including and excluding invalid assays. A graphical representation of the ACA distribution in histograms is provided in Figure 1.

Laboratory 2 performed 4 assays, each utilising only 1 vial of each test sample tested in duplicate. Laboratory 13 reported results from 5 assays, i.e. assays 1a, 1b, 2a, 2b and 3; for technical reasons assays 1a, 1b, 2a and 2b were performed using only 1 vial of each test sample and 1 vial of reference preparation (sample E) per assay. In assay 3, 2 vials of each test sample were tested concomitantly. Laboratory 16 reported results from only 1 assay.

The Ph. Eur. chapter 2.6.17 requires the complement control plot to show a straight line between 15 % and 85 % haemolysis and its slope to be between 0.15 and 0.40, preferably between 0.18 and 0.30. Deviations from the preferred range in the chapter are indicated on a shaded background in Table 5. They are, however, valid with regard to the monograph's requirements and were thus included in the overall calculation.

Table 4 – *Results of the Fc function test on Day 1 (central calculations)*

Lab	Assay	Vial	Mean corrected slope				Index of Fc function	
			Control	Sample A	Sample B	Sample F	A versus F	B versus F
2	1	1	0,001	0,089	0,090	0,094	94,5	95,3
		2	0,001	0,090	0,088	0,094	95,8	93,3
	2	1	0,003	0,087	0,088	0,087	99,3	100,1
		2	0,003	0,087	0,087	0,087	100,0	99,8
	3	1	0,004	0,088	0,089	0,097	90,4	91,9
		2	0,004	0,091	0,088	0,097	93,7	90,8
3	1	1	0,053	0,219	0,214	0,213	104,0	100,6
		2	0,053	0,211	0,219	0,213	98,7	103,9
	2	1	0,050	0,226	0,238	0,205	113,5	121,2
		2	0,050	0,232	0,237	0,205	117,0	120,1
	3	1	0,099	0,203	0,216	0,206	97,4	109,6
		2	0,099	0,213	0,222	0,206	107,0	115,3
8	1	1	0,018	0,393	0,377	0,368	106,9	102,6
		2	0,018	0,390	0,373	0,368	106,2	101,3
	2	1	0,021	0,426	0,409	0,399	107,3	102,6
		2	0,021	0,415	0,409	0,399	104,3	102,7
	3	1	0,014	0,349	0,352	0,340	102,6	103,7
		2	0,014	0,349	0,346	0,340	102,8	101,7
13	1	1	0,037	0,159	n.t.	0,151	107,0	n.t.
			0,013	n.t.	0,114	0,154	n.t.	71,9
		2	0,041	0,157	n.t.	0,178	84,8	n.t.
			0,043	n.t.	0,183	0,204	n.t.	87,2
	2	1	0,044	0,164	n.t.	0,157	106,0	n.t.
			0,030	n.t.	0,163	0,172	n.t.	93,6
		2	0,039	0,184	n.t.	0,213	83,1	n.t.
			0,035	n.t.	0,190	0,218	n.t.	84,5
	3	1	0,035	0,159	n.t.	0,159	100,5	n.t.
			0,010	n.t.	0,114	0,154	n.t.	72,3
		2	0,033	0,168	n.t.	0,203	79,3	n.t.
			0,031	n.t.	0,164	0,215	n.t.	72,5
14	1	1	-0,031	-0,155	-0,147	-0,159	96,3	90,4
		2	-0,031	-0,160	-0,154	-0,159	100,4	96,0
	2	1	0,029	-0,127	-0,124	-0,144	90,1	88,4
		2	0,029	-0,124	-0,142	-0,144	88,0	98,8
	3	1	0,016	-0,107	-0,109	-0,140	78,3	79,7
		2	0,016	-0,101	-0,115	-0,140	75,0	83,9
17	1	1	-0,045	-0,346	-0,333	-0,310	113,4	108,5
		2	-0,045	-0,338	-0,326	-0,310	110,4	106,0
	2	1	-0,068	-0,378	-0,372	-0,368	103,5	101,3
		2	-0,068	-0,370	-0,383	-0,368	100,9	105,1
	3	1	-0,072	-0,362	-0,375	-0,347	105,6	110,4
		2	-0,072	-0,357	-0,385	-0,347	104,0	113,8
						N	6	6
						Mean	99,1	97,8
						SD	7,8	11,8
						RSD	7,9	12,0

Lab: laboratory; N: number of laboratories; n.t.: not tested; RSD: relative standard deviation; SD: standard deviation.

Table 5 – *Results of the ACA test on Day 1 (central calculations)*

			Compl. Control		Sample E						Sample C				Sample D			
					Negative		Positive				Negative		Positive		Negative		Positive	
Lab	Assay	Repl.	Mean activity (CH50/mL)	Slope	% Act	Slope	% Act	Slope	Vial	Repl.	% Act	Slope	% Act	Slope	% Act	Slope	% Act	Slope
2	1	1	88,3	0,214	19,7%	0,228	76,5%	0,209	1	1	24,6%	0,302	91,4%	0,268	25,9%	0,248	91,2%	0,229
		2	89,7	0,230	19,8%	0,255	76,1%	0,215		2	25,7%	0,302	91,3%	0,246	25,8%	0,248	91,4%	0,234
	2	1	87,9	0,186	23,1%	0,172	73,9%	0,198	1	1	27,4%	0,175	Inv	Inv	28,2%	0,181	Inv	Inv
		2	86,9	0,174	22,1%	0,165	74,2%	0,202		2	26,2%	0,170	Inv	Inv	25,6%	0,198	Inv	Inv
	3*	1	110,0	0,335	20,5%	0,385	67,2%	0,237	1	1	32,9%	0,308	89,5%	0,295	30,9%	0,248	88,8%	0,289
		2	107,2	0,391	22,1%	0,383	61,1%	***0,416***		2	28,6%	0,329	89,2%	0,235	30,9%	0,274	89,0%	0,244
	4	1	96,2	0,174	23,1%	0,189	67,3%	0,203	1	1	28,7%	0,214	Inv	Inv	26,9%	0,192	Inv	Inv
		2	96,6	0,170	21,9%	0,189	71,1%	0,201		2	26,8%	0,204	Inv	Inv	27,3%	0,200	Inv	Inv
3	1	1	94,0	0,194	16,20%	0,201	82,4%	0,248	1	1	19,2%	0,202	94,2%	0,294	19,7%	0,201	94,6%	0,306
										2	19,3%	0,202	94,2%	0,292	19,9%	0,199	94,6%	0,306
		2	93,9	0,195	16,2%	0,201	82,4%	0,248	2	1	19,9%	0,214	94,2%	0,292	19,4%	0,208	94,3%	0,295
										2	20,0%	0,214	94,2%	0,291	19,5%	0,209	94,3%	0,295
	2	1	92,4	0,197	17,7%	0,221	77,9%	0,278	1	1	18,6%	0,220	94,2%	0,302	18,5%	0,210	94,6%	0,316
										2	18,6%	0,220	94,3%	0,303	18,6%	0,210	94,6%	0,315
		2	92,4	0,197	17,8%	0,220	78,0%	0,278	2	1	21,1%	0,229	94,2%	0,297	20,7%	0,220	94,4%	0,304
										2	21,1%	0,229	94,2%	0,297	20,7%	0,220	94,4%	0,304
	3	1	91,5	0,210	16,2%	0,212	79,7%	0,247	1	1	16,9%	0,210	93,8%	0,291	16,9%	0,196	94,5%	0,310
										2	17,0%	0,210	93,9%	0,291	16,9%	0,196	94,5%	0,309
		2	91,4	0,211	16,2%	0,212	79,7%	0,247	2	1	15,9%	0,217	93,9%	0,295	18,2%	0,198	94,3%	0,302
										2	15,9%	0,218	93,9%	0,295	18,2%	0,198	94,3%	0,303
5	1	1	99,0	0,163	13,1%	0,177	75,7%	0,232	1	1	20,1%	0,171	86,6%	0,249	18,7%	0,170	81,4%	0,237
										2	20,2%	0,171	87,0%	0,260	17,7%	0,168	85,4%	0,248
		2	99,4	0,163	11,9%	0,173	74,3%	0,231	2	1	19,6%	0,172	86,5%	0,257	18,5%	0,172	89,3%	0,285
										2	19,6%	0,172	85,5%	0,249	17,3%	0,168	88,8%	0,273
	2	1	103,3	0,158	13,7%	0,164	73,1%	0,204	1	1	20,3%	0,173	74,2%	0,214	19,3%	0,173	85,2%	0,251
										2	20,2%	0,166	76,9%	0,225	19,4%	0,176	79,2%	0,251
		2	103,8	0,157	11,3%	0,183	76,2%	0,239	2	1	20,0%	0,172	81,5%	0,226	18,7%	0,180	81,7%	0,249
										2	19,6%	0,175	83,7%	0,250	18,6%	0,179	88,6%	0,287
	3	1	103,0	0,160	12,4%	0,199	75,9%	0,247	1	1	19,3%	0,174	92,0%	0,331	19,3%	0,173	91,7%	0,338
										2	19,6%	0,171	91,3%	0,312	19,3%	0,167	91,8%	0,342
		2	101,2	0,165	10,9%	0,199	75,5%	0,241	2	1	18,0%	0,185	90,7%	0,343	15,9%	0,209	90,8%	0,361
										2	17,7%	0,180	88,7%	0,320	14,6%	0,218	90,6%	0,359
13	1a	1	107,8	0,197	29,8%	0,222	69,6%	0,253	1	1	33,9%	0,253	92,0%	***0,452***	31,7%	0,216	90,8%	0,383
		2	109,3	0,199	21,4%	0,217	74,6%	0,253		2	33,8%	0,231	Inv	Inv	35,2%	0,223	Inv	Inv
	1b	1	111,2	0,193	25,8%	0,196	60,0%	0,244	2	1	40,3%	0,222	89,2%	0,298	39,3%	0,215	Inv	Inv
		2	114,7	0,186	33,8%	0,204	66,5%	0,235		2	40,9%	0,213	89,3%	0,316	44,0%	0,222	90,3%	0,312
	2a	1	81,5	0,480	27,3%	***0,496***	71,4%	***0,497***	1	1	32,1%	***0,509***	93,9%	***0,431***	29,7%	***0,516***	Inv	Inv
		2	84,6	0,476	27,6%	***0,472***	71,4%	***0,497***		2	30,9%	***0,522***	Inv	Inv	31,6%	***0,513***	92,5%	***0,418***
	2b	1	82,5	0,454	28,6%	***0,460***	64,0%	***0,486***	2	1	37,5%	***0,487***	88,6%	***0,448***	34,8%	***0,515***	89,5%	***0,429***
		2	85,0	0,444	30,9%	***0,511***	73,5%	***0,451***		2	40,8%	***0,463***	91,2%	***0,426***	39,8%	***0,496***	89,9%	***0,431***
	3	1	93,9	0,373	20,0%	***0,408***	69,9%	0,380	1	1	22,5%	0,388	90,3%	0,362	22,5%	***0,402***	91,0%	0,364
										2	27,5%	0,384	90,9%	0,379	24,7%	***0,406***	90,7%	0,375
		2	93,9	0,373	22,5%	0,383	71,6%	0,387	2	1	23,7%	***0,413***	82,1%	***0,403***	29,3%	***0,419***	86,4%	***0,408***
										2	27,5%	***0,412***	88,1%	0,390	29,8%	***0,407***	89,6%	0,370
14	1	1	*87,1*	*0,200*	*12,3%*	*0,197*	49,7%	*0,244*	1	1	*19,0%*	*0,202*	*84,1%*	*0,263*	*17,8%*	*0,208*	*84,4%*	*0,269*
										2	*17,2%*	*0,205*	*83,3%*	*0,271*	*17,6%*	*0,206*	*83,7%*	*0,265*
		2	*89,7*	*0,193*	*14,2%*	*0,193*	52,2%	*0,230*	2	1	*18,5%*	*0,203*	*83,2%*	*0,269*	*19,4%*	*0,207*	*84,4%*	*0,282*
										2	*18,9%*	*0,207*	*83,1%*	*0,267*	*17,9%*	*0,203*	*67,8%*	*0,207*
	2	1	*83,0*	*0,195*	*17,9%*	*0,225*	55,2%	*0,242*	1	1	*24,1%*	*0,224*	*58,6%*	*0,231*	*23,3%*	*0,225*	*57,9%*	*0,231*
										2	*23,8%*	*0,218*	*58,5%*	*0,234*	*24,2%*	*0,221*	*57,7%*	*0,230*
		2	*82,1*	*0,198*	*21,9%*	*0,227*	55,2%	*0,243*	2	1	*23,4%*	*0,225*	*58,1%*	*0,232*	*23,4%*	*0,224*	*57,7%*	*0,233*
										2	*24,5%*	*0,225*	*57,8%*	*0,234*	*23,7%*	*0,222*	*58,3%*	*0,226*
	3	1	*92,2*	*0,201*	*20,1%*	*0,219*	49,2%	*0,249*	1	1	*23,8%*	*0,220*	*82,3%*	*0,256*	*24,2%*	*0,218*	*81,9%*	*0,255*
										2	*23,0%*	*0,221*	*80,9%*	*0,256*	*25,4%*	*0,218*	*80,2%*	*0,265*
		2	*91,3*	*0,205*	*21,7%*	*0,221*	48,4%	*0,215*	2	1	*23,5%*	*0,221*	*82,2%*	*0,257*	*23,2%*	*0,219*	*81,9%*	*0,256*
										2	*24,8%*	*0,216*	*81,3%*	*0,261*	*21,6%*	*0,219*	*80,1%*	*0,205*
16	1	1	*104,4*	*0,234*	6,8%	*0,236*	*61,5%*	*0,236*	1	1	*15,6%*	*0,250*	*75,2%*	*0,236*	*15,0%*	*0,249*	*71,3%*	*0,222*
										2	*14,6%*	*0,255*	*74,6%*	*0,245*	*14,1%*	*0,246*	*70,4%*	*0,235*
		2	*103,7*	*0,247*	5,7%	*0,237*	*61,1%*	*0,234*	2	1	*16,4%*	*0,249*	*71,9%*	*0,230*	*15,3%*	*0,244*	*72,7%*	*0,237*
										2	*15,3%*	*0,245*	*71,2%*	*0,241*	*14,3%*	*0,245*	*72,0%*	*0,237*
17	1	1	90,8	0,355	15,5%	0,359	67,7%	0,340	1	1	18,8%	0,367	94,3%	***0,435***	17,7%	0,360	93,5%	0,388
										2	21,2%	0,360	94,0%	***0,422***	17,4%	0,363	94,1%	***0,440***
		2	91,8	0,338	17,0%	0,369	67,8%	0,331	2	1	18,3%	0,361	94,2%	***0,434***	15,9%	0,362	94,3%	***0,447***
										2	18,1%	0,358	93,7%	***0,449***	17,3%	0,353	94,1%	***0,430***
	2	1	89,0	0,383	15,0%	0,393	61,2%	0,336	1	1	18,0%	0,382	92,1%	***0,418***	15,7%	0,377	93,2%	***0,454***
										2	19,4%	0,388	91,8%	***0,445***	15,4%	0,371	92,1%	0,387
		2	87,3	0,381	14,5%	0,384	62,6%	0,330	2	1	17,6%	0,378	90,2%	***0,427***	14,9%	0,375	90,7%	***0,425***
										2	16,8%	0,376	91,5%	***0,431***	14,8%	0,379	89,8%	***0,438***
	3	1	98,4	0,360	14,7%	0,359	69,9%	0,312	1	1	17,6%	0,349	91,3%	0,386	15,6%	0,333	90,5%	0,389
										2	16,3%	0,343	93,1%	0,385	16,7%	0,348	93,1%	0,352
		2	98,2	0,333	13,9%	0,335	70,2%	0,301	2	1	13,0%	0,327	93,0%	0,363	15,3%	0,326	93,5%	0,378
										2	13,4%	0,330	92,7%	0,374	14,1%	0,328	93,5%	0,385
Including all tests			N	7		7					7		7		7		7	
			Mean	16,7%		67,8%					21,9%		85,7%		21,4%		85,6%	
			SD	6,56		9,31					6,13		8,54		6,57		9,36	
Excluding invalid tests			N	5		5					5		5		5		5	
			Mean	18,4%		72,6%					23,3%		90,4%		23,6%		91,0%	
			SD	5,70		5,32					6,84		3,32		8,96		2,81	

Values causing the test to be invalid according to Ph. Eur. chapter 2.6.17 are printed on a dark background.
Corresponding calculated results are indicated in italics. Shaded cells show valid slopes outside the preferred range (0.18-0.30). Slopes outside the 0.15-0.40 range are in bold italics.
Inv: absence of a straight line between 15 % and 85 % haemolysis. Act: activity; Compl: complement; Lab: laboratory; N: number of laboratories; Repl: replicate; SD: standard deviation.
* This assay was considered invalid by the participant due to high haemolysis in some tubes.

The mean activity of the complement control was within the required range of 80-120 CH_{50}/mL in all assays. Assays for which the complement control slope was outside the required range of 0.15-0.40 were considered invalid. These are indicated on a dark background in Table 5. Likewise, assays which did not show a straight line between 15 % and 85 % haemolysis were also considered invalid as prescribed in the general chapter. In some cases, even if the complement control plot was within the required limits, some of the samples showed slopes outside the required range. These individual samples, shown in bold italics in Table 5, were also considered invalid. Invalid assays were excluded from the overall calculations.

The current BRP (sample E) leaflet requires 10-40 % complement consumption for the negative control and 60-100 % for the positive control. For this study, this requirement was not used as a validity criterion when applied to the cBRPs, but rather as a measure of suitability of the proposed candidate standards. In contrast, assays for which these criteria were not met for the reference preparation, potentially indicating a technical problem, were considered invalid. This was the case for all assays by laboratories 14 and 16. They are presented on a dark background in Table 5.

Overall calculations were performed including all assays and samples, and excluding invalid samples and invalid assays, as appropriate. The overall mean for the BRP, excluding invalid assays, was 18.4 % and 72.6 % for the negative and positive controls, respectively. Overall ACA means were very similar for both candidate materials: i.e. 23.3 % and 90.4 % for sample C and 23.6 % and 91.0 % for sample D for the negative and positive controls, respectively. Excluding invalid results led to slightly, but not significantly, higher mean ACA for all samples; however, variability was reduced in most cases (Table 5). As the vast majority of assays fell within the currently required ranges for ACA, the candidate materials were considered suitable for the intended purpose.

In light of the results obtained, the ACA ranges required for positive and negative controls prepared with the BRP could be maintained as they were for the previous BRP batches; however, the positive control sample volume had to be revised to 0.8 mL instead of 0.6 mL.

4.3. Distribution of molecular size

All 17 laboratories performed the assay for distribution of molecular size. The results are listed in Table 6 and general information on the methods used by the participants is given in Table 3. Laboratory 3 performed tests at 2 different sites. They were considered herein as 2 different laboratories and thus treated as independent assays. Laboratory 4 performed the assay using various protein concentrations and injection volumes; more details can be found in Table 3. Although they are distinguished herein as versions 1, 2 and 3, it appears that all 3 versions yielded similar results (Table 6).

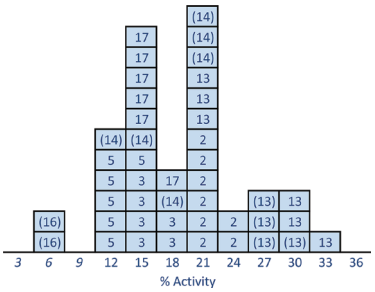
The relative retention for the dimer peak versus the monomer peak was calculated by participants for every sample and vial. In addition, the retention times of the cBRPs for both peaks were compared to those obtained with the BRP (sample E). The latter calculations were carried out centrally.

The RR of dimer to monomer ranged from 0.834 to 0.890, with mean ratio values of 0.856 for both candidate materials and for the BRP (sample E); this was in line with the RR of around 0.85 indicated in the monographs. Reproducibility was satisfactory with a relative standard deviation (RSD) ranging from 1.08 to 1.26 %.

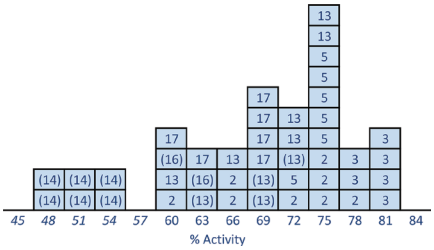
In addition, the monographs require that, ‘for the monomer and for the dimer, the retention time relative to the corresponding peak in the chromatogram obtained with the reference solution is 1 ± 0.02’. This parameter was also assessed (Table 6) and the criterion was fulfilled for both cBRPs in all assays. Both candidate materials appeared virtually identical to the BRP with overall values above or equal to 0.999. Reproducibility was also very good for this parameter, with the overall RSD in the range of 0.08-0.31 %.

Figure 1 – *Distribution of ACA on Day 1 (central calculations)*

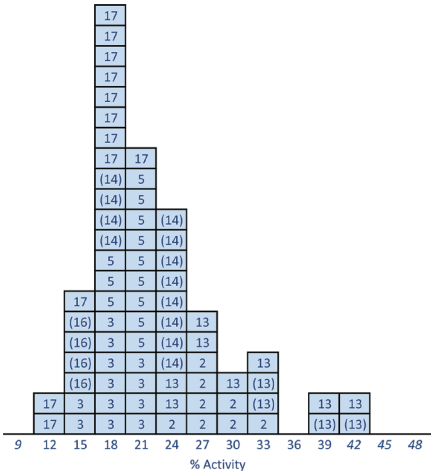
Sample E: negative control



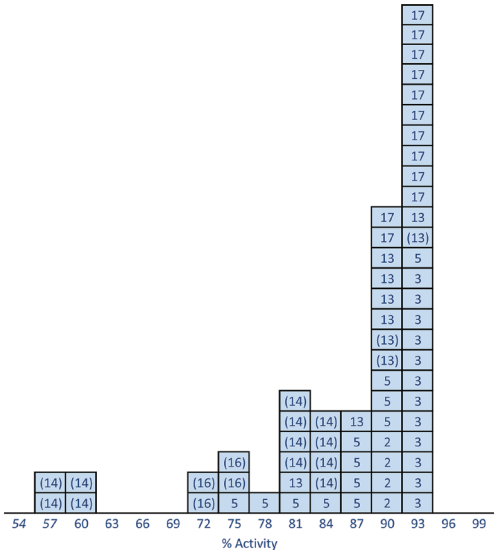
Sample E: positive control



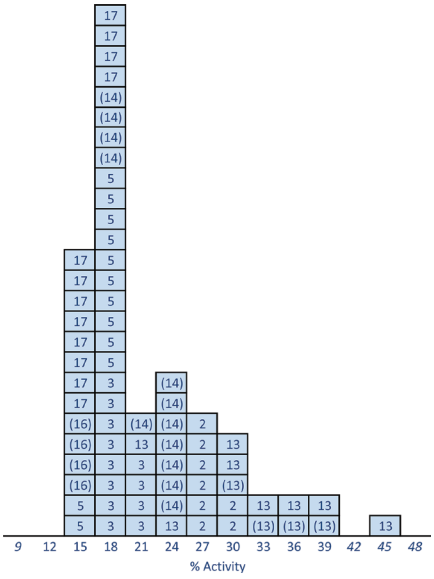
Sample C: negative control



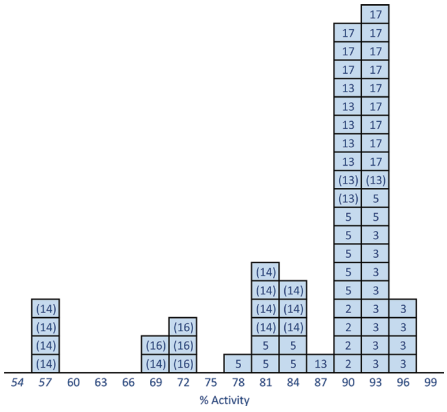
Sample C: positive control



Sample D: negative control



Sample D: positive control



Numbers represent laboratory codes. Invalid assays according to Ph. Eur. chapter 2.6.17 requirements are indicated in parentheses. Numbers in italics indicate values outside the requirements of the monographs [2-4]. The corresponding assays were considered invalid.

5. STABILITY OF THE cBRPS

5.1. Short-term stability assessment

As the content of each vial largely exceeds the amount of material necessary for 1 assay, participants were asked to store opened vials (samples A and B) or reconstituted vials (samples C and D) for up to 14 days at 2-8 °C to assess their stability under these conditions. In all tests, the stored samples were compared to fresh vials of the corresponding BRP. The results of central calculations are presented in Tables 7, 8 and 9 for the Fc function, ACA and molecular size tests, respectively. Calculations returned by participants are given in the appendix in separate tables.

5.1.1. Fc function

Five laboratories provided results for Days 7 and 14. Laboratory 13 performed 3 assays on Days 7 and 14, testing the control and sample F for every vial as on Day 1.

All results from Day 7 were valid. The overall mean IFc was 102.6 % and 100.8 % for samples A and B versus the BRP, respectively. On Day 14, all assays were valid except for 1 assay for sample B by laboratory 3, for which unexpectedly high values were observed, i.e. 140.7 % and 142.7 % (Table 7). These values were determined as being statistical outliers using the interquartile range method, i.e. both values were at the limit or higher than 3 interquartiles above the third quartile (Figure 2) and were thus excluded from the overall calculation. The reasons for this discrepancy are not clear but as all other parameters were satisfactory, it may be considered that the issue was linked to this particular assay rather than to a stability problem in general. For 1 vial of sample A (laboratory 13, assay 1, vial 2), the observed IFc was slightly lower than 60 %. However, this value could not be excluded as an outlier and was thus included in the overall estimation. The overall mean IFc was 96.6 % for sample A and 89.6 % for sample B (excluding invalid results).

The IFc, calculated relative to freshly opened BRP, was satisfactory for up to 14 days of storage. The inter-assay variability was also generally satisfactory. Based on these results, it can be considered that the cBRPs are stable and can be used for this test for up to 14 days if stored at 2-8 °C after their first opening, provided precautions are taken to avoid contamination. It is thus recommended to open vials under a laminar flow using gloves, and to use sterile materials and reagents.

5.1.2. Anticomplementary activity

A total of 5 laboratories provided results for the assessment of ACA on Days 7 and 14 (Day 15 for laboratory 5). The mean results per laboratory were calculated (Table 8) and the means of means are shown at the bottom of Table 8. The validity criteria considered for Day 1 were also applied here. On Day 7, assay 1a by laboratory 13 exhibited a slope outside the accepted range (0.15-0.40) for the complement control. For Laboratory 13 in assay 2a and for laboratory 14, the activity of the positive control using the BRP was below the required limit of 60 %. These 3 assays were thus considered invalid. For both days, values for the complement control were within the range of 80-120 CH_{50}/mL required by the monograph in all cases except one i.e. Day 14 laboratory 14, which was just below the lower limit. In this assay, the activity of the BRP positive control was also below the lower limit. It was thus considered invalid as well.

Overall, only marginal changes in ACA were observed for all samples, including the reference preparation (mainly a slight increase for the negative controls) during the observation period (14 days). The values obtained were still well within the limits stated in the BRP leaflet for both positive and negative controls. Based on this, the stability of samples C and D under these conditions of storage, i.e. at 2-8 °C, appeared satisfactory. Therefore, if necessary, the BRPs may be used for the ACA assay for up to 14 days after reconstitution, subject to the restrictions mentioned in section 5.1.1 regarding precautions for use.

Table 6 – *Summary of relative retentions obtained by SEC on Day 1 (central calculations)*

		Dimer/Monomer					A versus E		B versus E	
		Sample A		Sample B						
Lab	Assay	Vial 1	Vial 2	Vial 1	Vial 2	Sample E	monomer	dimer	monomer	dimer
1	1	0,835	0,835	0,835	0,834	0,834	1,000	1,000	1,000	1,000
	2	0,834	0,834	0,834	0,834	0,834	1,000	1,000	1,000	1,000
2	1	0,852	0,852	0,852	0,852	0,852	1,000	0,999	1,000	0,999
	2	0,852	0,852	0,852	0,852	0,852	1,000	0,999	1,000	0,999
3a	1	0,855	0,855	0,855	0,855	0,855	1,000	1,000	1,000	1,000
	2	0,854	0,854	0,854	0,854	0,854	0,999	1,000	0,999	1,000
3b	1	0,857	0,857	0,857	0,857	0,858	1,000	0,999	1,001	0,999
	2	0,856	0,856	0,857	0,858	0,851	0,995	1,001	0,993	1,001
4 Version 1	1	0,845	0,845	0,845	0,845	0,846	1,000	0,999	0,999	0,998
	2	0,845	0,844	0,845	0,845	0,846	0,999	0,998	0,999	0,998
4 Version 2	1	0,846	0,846	0,846	0,846	0,845	0,998	0,998	0,997	0,997
	2	0,845	0,845	0,845	0,845	0,845	0,998	0,998	0,998	0,998
4 Version 3	1	0,845	0,846	0,845	0,845	0,846	0,999	0,998	0,998	0,997
	2	0,845	0,845	0,845	0,846	0,846	0,999	0,998	0,999	0,998
5	1	0,850	0,851	0,851	0,851	0,850	1,000	1,000	1,000	1,000
	2	0,850	0,850	0,850	0,850	0,850	1,000	1,000	1,000	1,000
6	1	0,848	0,848	0,847	0,848	0,848	0,998	0,998	1,001	1,001
	2	0,847	0,847	0,847	0,846	0,847	1,000	1,000	1,001	1,001
7	1	0,863	0,863	0,862	0,863	0,861	0,999	1,001	0,999	1,001
	2	0,863	0,862	0,862	0,862	0,861	1,002	1,003	1,000	1,001
8	1	0,860	0,860	0,860	0,860	0,859	0,999	1,000	0,999	1,000
	2	0,860	0,860	0,860	0,860	0,859	1,000	1,001	1,000	1,001
9	1	0,865	0,865	0,865	0,865	0,865	1,000	1,000	1,000	1,000
	2	0,865	0,865	0,865	0,865	0,865	1,000	1,000	1,000	1,000
10	1	0,861	0,861	0,861	0,861	0,860	1,000	1,001	1,000	1,001
	2	0,861	0,861	0,861	0,861	0,860	1,000	1,000	1,000	1,000
11	1	0,860	0,860	0,860	0,860	0,860	0,999	0,999	0,998	0,998
	2	0,859	0,859	0,859	0,859	0,859	0,998	0,998	0,998	0,998
12	1	0,856	0,855	0,855	0,855	0,855	1,000	1,000	1,000	1,000
	2	0,855	0,855	0,855	0,855	0,856	1,000	1,000	1,000	1,000
13	1	0,856	0,856	0,856	0,856	0,855	1,000	1,001	1,000	1,001
	2	0,855	0,855	0,855	0,855	0,855	1,000	1,001	1,000	1,001
14	1	0,877	0,875	0,876	0,876	0,890	0,999	0,983	0,999	0,983
	2	0,877	0,876	0,876	0,879	0,883	0,999	0,993	0,999	0,993
15	1	0,854	0,854	0,854	0,853	0,854	1,000	1,000	1,001	1,001
	2	0,854	0,853	0,854	0,853	0,853	1,001	1,001	1,001	1,001
16	1	0,866	0,866	0,866	0,865	0,864	0,999	1,001	0,999	1,000
	2	0,865	0,865	0,865	0,865	0,864	0,999	1,000	0,999	1,000
	N	17		17		17	17	17	17	17
	Mean	0,856		0,856		0,856	0,999	0,999	1,000	0,999
	SD	0,009		0,009		0,011	0,001	0,003	0,001	0,003
	RSD	1,084		1,093		1,258	0,076	0,309	0,097	0,298

Lab: laboratory; N: number of laboratories; RSD: relative standard deviation; SD: standard deviation.
Three different methods were used by laboratory 4. See text for details.

5.1.3. Molecular size distribution by SEC

A total of 16 laboratories provided results for Days 7 and 14. Laboratory 16 performed 2 assays on both days. As on Day 1, laboratory 3 tested the samples at 2 different sites; these were thus considered here as independent tests. Results from both test days are summarised in Table 9.

Table 7 – *Short-term stability assessment – Fc function test (central calculations)*

Day 7							
Lab		Vial	Mean corrected slope				Index of Fc function	
			Control	Sample A	Sample B	Sample F	A versus F	B versus F
3		1	0,088	0,192	0,187	0,177	117,3	111,6
		2	0,088	0,180	0,192	0,177	104,0	116,6
8		1	0,017	0,371	0,386	0,350	106,2	110,8
		2	0,017	0,373	0,360	0,350	106,9	103,0
13	assay 1	1	0,038	0,203	n.t.	0,221	90,1	n.t.
			0,037	n.t.	0,221	0,229	n.t.	96,0
		2	0,046	0,208	n.t.	0,219	93,4	n.t.
			0,041	n.t.	0,221	0,220	n.t.	100,5
	assay 2	1	0,040	0,216	n.t.	0,204	107,7	n.t.
			0,039	n.t.	0,209	0,218	n.t.	95,4
		2	0,045	0,217	n.t.	0,222	97,5	n.t.
			0,042	n.t.	0,224	0,224	n.t.	99,7
	assay 3	1	0,040	0,210	n.t.	0,219	95,0	n.t.
			0,032	n.t.	0,213	0,218	n.t.	97,3
		2	0,030	0,187	n.t.	0,220	82,7	n.t.
			0,031	n.t.	0,200	0,211	n.t.	93,6
14		1	-0,024	-0,134	-0,116	-0,133	101,1	84,9
		2	-0,024	-0,124	-0,109	-0,133	91,8	78,2
17		1	-0,063	-0,364	-0,365	-0,354	103,5	103,8
		2	-0,063	-0,373	-0,369	-0,354	106,7	105,1
Including all tests						N	5	5
						Mean	102,6	100,8
						SD	6,9	12,4

Day 14							
Lab		Vial	Mean corrected slope				Index of Fc function	
			Control	Sample A	Sample B	Sample F	A versus F	B versus F
3		1	0,107	0,200	0,224	0,190	112,0	140,7
		2	0,107	0,205	0,226	0,190	117,9	142,7
8		1	0,022	0,371	0,374	0,371	100,1	101,0
		2	0,022	0,383	0,366	0,371	103,4	98,8
13	assay 1	1	0,038	0,142	n.t.	0,179	74,0	n.t.
			0,042	n.t.	0,146	0,186	n.t.	71,9
		2	0,036	0,126	n.t.	0,191	57,9	n.t.
			0,039	n.t.	0,176	0,193	n.t.	89,0
	assay 2	1	0,033	0,150	n.t.	0,186	76,1	n.t.
			0,031	n.t.	0,174	0,197	n.t.	86,1
		2	0,038	0,188	n.t.	0,199	93,5	n.t.
			0,027	n.t.	0,167	0,193	n.t.	84,3
	assay 3	1	0,034	0,171	n.t.	0,204	80,7	n.t.
			0,036	n.t.	0,184	0,202	n.t.	89,4
		2	0,034	0,158	n.t.	0,188	80,6	n.t.
			0,030	n.t.	0,147	0,153	n.t.	95,0
14		1	-0,024	-0,181	-0,152	-0,186	96,8	79,2
		2	-0,024	-0,161	-0,149	-0,186	84,8	76,9
17		1	-0,052	-0,286	-0,283	-0,296	96,0	94,7
		2	-0,052	-0,297	-0,283	-0,296	100,6	94,8
Including all tests						N	5	5
						Mean	96,6	100,1
						SD	13,9	24,7
Excluding invalid tests						N		4
						Mean		89,6
						SD		9,6

Lab: laboratory; N: number of laboratories, n.t.: not tested; SD: standard deviation.

The migration parameters of the cBRPs remained unaffected by storage at 2-8 °C for up to 14 days; the dimer/monomer RR appeared unchanged when compared to the results obtained on Day 1. In addition, the retention time of both the dimer and the monomer were very close or equal to 1 relative to the reference preparation (sample E). Inter-assay variability was generally low with RSDs ranging from 0.1 to 0.5 %.

The cBRPs can therefore be considered suitable for use in this test for peak identification as stated in the monographs [2-5] for up to 14 days after reconstitution or first opening when handled with the necessary precautions (see section 5.1.1).

Peak areas for monomer, dimer and polymers/aggregates were also reported by participants for Days 1, 7 and 14 to investigate whether storage would have an impact on their relative proportions. Overall means and RSDs were calculated at the EDQM. As can be seen in the tables provided in the appendix (given for information), the contents of each form were within the limits prescribed in the monographs on Day 1, and no changes, such as for example increased aggregation, could be detected after up to 14 days of storage.

The cBRPs were therefore also considered stable for this parameter over a period of 14 days when stored appropriately (see section 5.1.1). However, it must be kept in mind that these reference preparations are not intended for quantitative use.

Figure 2 – *Assessment of outlying IFc results for sample B (central calculations)*

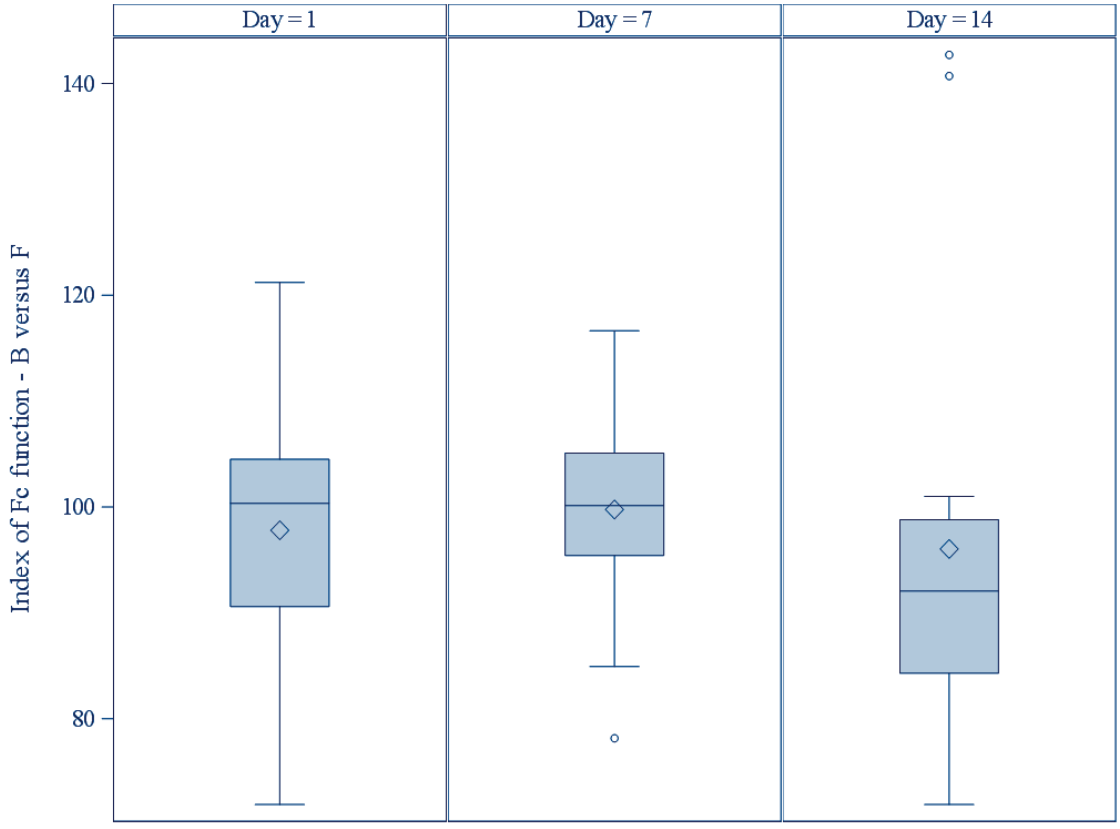


Table 8 – *Short-term stability assessment – ACA test (central calculations)*

Day 7																			
		Compl. Control			Sample E							Sample C				Sample D			
						Negative		Positive				Negative		Positive		Negative		Positive	
Lab	Assay	Repl.	Mean activity (CH50/mL)	Slope	Repl.	% Act	Slope	% Act	Slope	Vial	Repl.	% Act	Slope	% Act	Slope	% Act	Slope	% Act	Slope
3		1	96,4	0,200	1	14,1%	0,214	81,0%	0,253	1	1	15,9%	0,246	94,2%	0,305	15,3%	0,213	94,2%	0,309
										2	1	16,7%	0,228	94,8%	0,323	20,0%	0,226	94,2%	0,303
5		1	97,0	0,176	1	13,0%	0,320	74,5%	0,231	1	1	20,2%	0,263	90,6%	0,308	18,6%	0,271	90,4%	0,307
		2	90,8	0,303						2	1	22,7%	0,373	91,1%	0,315	18,8%	0,212	90,8%	0,311
13	1a	1	91,6	0,441	1	21,2%	0,392	74,6%	0,386	1	1	25,7%	***0,427***	87,0%	***0,403***	24,7%	0,381	83,8%	***0,429***
		2	96,4	0,441	2	24,9%	***0,412***	72,7%	***0,414***		2	30,2%	***0,405***	87,5%	***0,402***	31,1%	0,391	85,9%	***0,409***
	1b	1	108,8	0,192	1	26,1%	0,221	64,8%	0,240	1	1	28,8%	0,246	89,9%	0,283	28,8%	0,237	84,6%	0,252
		2	109,6	0,190	2	27,9%	0,232	69,2%	0,258		2	36,7%	0,249	90,6%	0,287	36,4%	0,246	89,3%	0,273
	2a	1	97,5	0,355	1	19,1%	0,385	50,8%	0,363	1	1	23,1%	0,383	90,3%	0,359	21,4%	0,386	90,3%	0,342
		2	100,5	0,343	2	21,4%	0,370	65,8%	0,365		2	27,5%	0,371	90,3%	0,359	27,1%	0,377	90,7%	0,362
	2b	1	99,2	0,367	1	21,7%	0,386	75,7%	0,382	1	1	23,5%	0,377	91,3%	0,387	26,4%	0,371	90,5%	0,366
		2	102,2	0,358	2	23,2%	0,371	77,4%	0,365		2	27,8%	0,368	92,0%	0,376	30,7%	0,368	88,5%	0,380
	3	1	93,5	0,279	1	26,4%	0,300	68,5%	0,300	1	1	36,7%	0,306	90,2%	0,292	n.t.	n.t.	89,0%	0,304
											2	35,5%	0,301	93,0%	0,312	43,5%	0,299	94,8%	0,398
		2	100,8	0,260	2	29,1%	0,296	67,4%	0,296	2	1	n.t.	n.t.	93,8%	0,305	23,9%	0,302	94,7%	0,390
											2	n.t.	n.t.	Inv	Inv	35,7%	0,306	Inv	Inv
14		1	83,1	0,187	1	19,1%	0,208	57,0%	0,249	1	1	25,3%	0,217	83,5%	0,265	25,0%	0,215	84,4%	0,271
		2	83,0	0,184						2	1	25,5%	0,216	84,1%	0,263	26,4%	0,218	85,1%	0,297
17		1	101,9	0,313	1	13,2%	0,332	69,2%	0,320	1	1	18,7%	0,343	89,2%	0,384	15,0%	0,320	91,6%	0,400
		2	100,8	0,312						2	1	16,5%	0,333	85,7%	0,372	15,1%	0,325	87,5%	0,383
Including all tests				N		5		5				5		5		5		5	
				Mean		16,7%		70,1%				22,1%		89,4%		21,4%		89,7%	
				SD		4,82		8,84				5,50		4,01		6,21		3,36	
Excluding invalid tests				N		4		4				4		4		4		4	
				Mean		16,4%		73,6%				21,6%		91,0%		20,7%		91,1%	
				SD		5,85		5,46				6,62		2,87		7,38		2,06	

Day 14																			
		Compl. Control			Sample E							Sample C				Sample D			
						Negative		Positive				Negative		Positive		Negative		Positive	
Lab	Assay	Repl.	Mean activity (CH50/mL)	Slope	Repl.	% Act	Slope	% Act	Slope	Vial	Repl.	% Act	Slope	% Act	Slope	% Act	Slope	% Act	Slope
3		1	91,3	0,279	1	22,2%	0,388	81,4%	0,291	1	1	16,8%	0,243	92,8%	0,252	20,1%	0,192	94,2%	0,274
		1			1					2	1	16,8%	0,245	92,8%	0,257	19,3%	0,230	94,1%	0,274
5		1	101,4	0,173	1	20,7%	0,185	73,7%	0,372	1	1	27,7%	0,205	76,8%	0,241	24,5%	0,210	70,5%	0,244
		2	99,3	0,175	2					2	1	24,5%	0,198	76,8%	0,262	24,8%	0,203	69,3%	0,293
13	1a	1	93,7	0,210	1	32,1%	0,244	66,5%	0,244	1	1	31,8%	0,252	92,8%	0,299	35,6%	0,283	91,7%	0,282
											2	36,4%	0,256	93,5%	0,277	41,2%	0,261	90,7%	0,273
	1b	2	98,7	0,212	2	30,4%	0,237	72,8%	0,251	2	1	40,5%	0,253	91,9%	0,276	35,9%	0,245	92,0%	0,287
		2			2						2	43,3%	0,259	92,9%	0,303	34,5%	0,260	94,0%	0,360
	2a	1	93,6	0,252	1	22,4%	0,291	65,4%	0,286	1	1	35,9%	0,296	92,5%	0,302	28,5%	0,299	91,1%	0,303
											2	37,3%	0,305	93,6%	0,322	36,1%	0,285	93,8%	0,346
	2b	2	103,8	0,243	2	25,3%	0,284	77,0%	0,300	2	1	27,1%	0,315	93,7%	0,338	28,5%	0,300	86,4%	0,299
					2						2	32,2%	0,297	92,4%	0,336	32,0%	0,299	93,3%	0,303
	3	1	111,8	0,205	1	26,8%	0,228	72,9%	0,234	1	1	26,8%	0,228	84,4%	0,246	29,8%	0,219	91,4%	0,253
											2	34,0%	0,231	79,7%	0,249	32,8%	0,210	92,4%	0,276
		2	116,6	0,210	2	26,1%	0,226	77,9%	0,231	2	1	34,7%	0,245	80,5%	0,258	26,1%	0,241	91,4%	0,264
		2			2						2	35,6%	0,245	91,9%	0,270	30,9%	0,202	92,4%	0,275
14		1	79,4	0,191	1	12,8%	0,204	48,1%	0,234	1	1	22,8%	0,199	82,2%	0,277	22,3%	0,203	82,3%	0,261
		2	82,1	0,181						2	1	23,0%	0,200	83,3%	0,324	23,0%	0,199	82,4%	0,257
17		1	99,1	0,315	1	15,9%	0,335	66,9%	0,286	1	1	22,3%	0,324	92,6%	0,384	21,3%	0,330	94,1%	0,353
		2	99,0	0,316						2	1	22,1%	0,320	91,7%	0,361	22,5%	0,340	93,8%	0,377
Including all tests				N		5		5				5		5		5		5	
				Mean		19,7%		68,4%				24,5%		86,9%		24,3%		86,4%	
				SD		5,61		12,49				6,56		6,92		4,98		10,40	
Excluding invalid tests				N		4		4				4		4		4		4	
				Mean		21,5%		73,5%				24,9%		87,9%		24,7%		87,4%	
				SD		4,66		5,99				7,49		7,53		5,65		11,72	

Values causing the test to be invalid according to Ph. Eur. chapter 2.6.17 are printed on a dark background. Corresponding calculated results are indicated in italics. Shaded cells show valid slopes outside the preferred range (0.18-0.30). Slopes outside the 0.15-0.40 range are in bold italics.
Inv: absence of a straight line between 15 % and 85 % haemolysis.
Act: activity; Comp: complement; Lab: laboratory; n.t.: not tested; N: number of laboratories, Repl: replicate; SD: standard deviation.

Table 9 – *Short-term stability study – Molecular size distribution test by SEC (central calculations)*

Day 7										
Lab	Assay	Relative retention Dimer/Monomer					A versus E		B versus E	
		Sample A		Sample B		Sample E				
		Vial 1	Vial 2	Vial 1	Vial 2		monomer	dimer	monomer	dimer
1		0,834	0,834	0,834	0,834	0,834	1,000	1,001	1,000	1,001
3a		0,855	0,855	0,855	0,855	0,854	1,000	1,000	1,000	1,000
3b		0,856	0,856	0,856	0,856	0,851	0,996	1,002	0,996	1,001
4 version 1		0,845	0,845	0,845	0,845	0,845	0,999	0,998	0,999	0,998
4 version 2		0,845	0,845	0,845	0,845	0,845	0,999	0,998	0,999	0,998
4 version 3		0,845	0,845	0,845	0,845	0,846	0,999	0,998	0,999	0,998
5		0,850	0,850	0,850	0,850	0,850	1,000	1,000	1,000	1,000
6		0,847	0,847	0,847	0,846	n.r.	n.a.	n.a.	n.a.	n.a.
7		0,863	0,862	0,863	0,862	0,861	1,000	1,002	1,000	1,002
8		0,860	0,860	0,860	0,860	0,859	0,999	1,000	0,999	1,000
9		0,865	0,865	0,865	0,865	0,865	1,000	1,000	1,000	1,000
10		0,860	0,860	0,860	0,860	0,860	1,000	1,001	1,000	1,001
11		0,861	0,861	0,860	0,860	0,861	0,998	0,999	0,998	0,998
12		0,855	0,855	0,855	0,855	0,855	1,000	1,000	1,000	1,000
13		0,855	0,855	0,855	0,855	0,854	1,001	1,002	1,000	1,001
14		0,876	0,878	0,877	0,877	0,883	0,999	0,993	1,004	0,997
15		0,854	0,854	0,854	0,854	0,853	1,000	1,001	1,000	1,000
16	1	0,865	0,866	0,866	0,866	0,865	1,002	1,002	1,000	1,001
16	2	0,866	0,866	0,866	0,866	0,865	1,005	1,006	1,001	1,002
N		16		16		15	15	15	15	15
Mean		0,856		0,856		0,857	1,000	1,000	1,000	1,000
SD		0,010		0,010		0,011	0,001	0,002	0,002	0,001
RSD		1,158		1,162		1,258	0,146	0,244	0,169	0,124

Day 14										
Lab	Assay	Relative retention Dimer/Monomer					A versus E		B versus E	
		Sample A		Sample B		Sample E				
		Vial 1	Vial 2	Vial 1	Vial 2		monomer	dimer	monomer	dimer
1		0,834	0,834	0,833	0,833	0,833	1,000	1,000	1,000	1,000
3a		0,854	0,854	0,854	0,854	0,854	1,000	1,000	1,000	1,000
3b		0,855	0,855	0,855	0,855	0,853	0,996	0,999	0,996	0,999
4 version 1		0,844	0,844	0,844	0,844	0,845	1,000	1,000	1,001	1,000
4 version 2		0,844	0,844	0,844	0,844	0,845	1,000	0,999	1,000	0,999
4 version 3		0,845	0,845	0,844	0,844	0,845	1,000	1,000	1,001	0,999
5		0,849	0,850	0,849	0,849	0,849	0,999	1,000	0,999	1,000
6		0,846	0,846	0,846	0,846	0,846	1,002	1,002	0,999	1,000
7		0,863	0,862	0,861	0,862	0,861	0,999	1,000	0,998	0,999
8		0,860	0,859	0,859	0,859	0,858	1,000	1,001	0,999	1,000
9		0,865	0,865	0,865	0,865	0,865	1,000	1,000	1,000	1,000
10		0,860	0,860	0,860	0,860	0,860	1,000	1,001	1,000	1,001
11		0,860	0,860	0,860	0,860	0,860	0,998	0,998	0,998	0,998
12		0,854	0,854	0,854	0,854	0,854	1,000	1,000	1,000	1,000
13		0,858	0,858	0,858	0,858	0,857	1,000	1,001	0,999	1,000
14		0,877	0,877	0,878	0,876	0,881	0,999	0,995	0,999	0,994
15		0,850	0,849	0,850	0,850	0,852	0,984	0,981	0,985	0,983
16	1	0,864	0,864	n.t.	n.t.	0,862	0,996	0,998	n.t.	n.t.
16	2	0,864	0,864	0,867	0,864	0,862	0,995	0,998	0,994	0,998
N		16		16		16	16	16	16	16
Mean		0,855		0,855		0,856	0,998	0,999	0,998	0,998
SD		0,010		0,010		0,010	0,004	0,005	0,004	0,004
RSD		1,182		1,187		1,213	0,407	0,493	0,376	0,445

N: number of laboratories; n.a.: not applicable; n.r. not reported; SD: standard deviation; RSD: relative standard deviation. Three different methods were used by laboratory 4. See text for details.

5.2. Long-term stability

Immunoglobulin preparations are generally very stable when stored appropriately, especially in lyophilised form. For samples A and B, data generated by the manufacturer on similar therapeutic products demonstrated that the preparation is stable for a minimum of 42 months when stored at the recommended temperature, i.e. + 5 °C.

With regard to samples C and D, stability data generated by the manufacturer – and past experience with BRPs prepared with similar products from the same manufacturer – showed

that their long-term stability was very good at the recommended storage temperature, i.e. + 5 °C. It was thus deemed unnecessary to perform an accelerated degradation study for these candidate materials. Nevertheless, the BRPs, once adopted, will be monitored by SEC at regular intervals throughout their lifetime.

6. CONCLUSIONS

The study results demonstrated that:

1. Sample A is suitable as a reference preparation for the Fc function test and for the molecular size determination test by SEC as required by the monographs [2-5]. The batch of candidate material was split into 2 parts, each dedicated specifically to one or the other test. They were submitted to the Ph. Eur. Commission in June 2017 for adoption as Ph. Eur. human immunoglobulin for Fc function BRP batch 1 and Ph. Eur. human immunoglobulin (molecular size) BRP batch 2.
2. Sample B is suitable as a reference preparation for the Fc function test and for the molecular size determination test by SEC as required by the monographs [2-5]. The candidate BRP was divided into 2 parts as described above. They were submitted to the Ph. Eur. Commission in June 2017 for adoption as Ph. Eur. human immunoglobulin for Fc function BRP batch 2 and Ph. Eur. human immunoglobulin (molecular size) BRP batch 3. These batches will be distributed when the corresponding batches 1 and 2 have been exhausted.

The IFc limits shall remain as required at present, i.e. the IFc of the test sample relative to that of the BRP should not be less than 60 %. Users of the BRPs are reminded that these are supplied in liquid form, unlike the previous batches.

3. Samples C and D are suitable as reference preparation for the ACA test as required by monograph 0918. They were submitted to the Ph. Eur. Commission in June 2017 for adoption as Ph. Eur. human immunoglobulin for anticomplementary activity BRP batch 1 (sample C) and batch 2 (sample D). Batch 2 will be distributed once batch 1 has been exhausted. For use in the test, the negative and positive controls are to be prepared from 0.2 mL and 0.8 mL of reconstituted BRP (50 mg/mL), respectively.

The above specifications for the individual tests are reiterated in the respective accompanying leaflets, as applicable. Leaflets can be downloaded from the EDQM website.[3] A revision of the general chapters [7, 8] was adopted by the Ph. Eur. Commission at its 156th session in November 2016 to introduce the new nomenclature and use of the new BRPs.

The BRPs are available from the EDQM under the following catalogue numbers:

- Y0001966 for the human immunoglobulin for Fc function BRP;
- Y0001994 for the human immunoglobulin for anticomplementary activity BRP;
- Y0000488 for the human immunoglobulin (molecular size) BRP.

The data generated by the manufacturers of the starting materials on similar batches, as well as historical data from previous BRPs prepared from similar materials, show that the cBRPs are stable at the recommended long-term storage temperature (+ 5 °C). They will nevertheless be monitored by SEC regularly throughout their lifetime. The BRPs will be shipped at + 5 °C for distribution to users.

The study also demonstrated that the BRPs can be considered stable for the intended purpose and may be used in all 3 tests, as applicable, for up to 14 days after first use or after reconstitution, provided they are reconstituted and/or opened aseptically, and stored at 2-8 °C.

3 https://www.edqm.eu/en/ph-eur-reference-standards-purpose-and-use.

7. ACKNOWLEDGEMENTS

The organisers express their sincere gratitude to the manufacturers, in particular LFB (France), for their kind donation of the starting materials, and to Dr D. Karra (PEI, Langen, Germany) for acting as project leader and for her support and advice throughout the project.

Thanks also go to all the participants and their colleagues/co-workers at the bench for their involvement in this project. Special thanks go to Ms S. Fromweiler and Ms S. Woodward for their skilful assistance.

This study was run by the EDQM in the framework of the Biological Standardisation Programme, a joint programme carried out with funding by the European Union and by the Council of Europe, under project code BSP122. This document was produced with financial assistance by the European Union. The views expressed herein can in no way be taken to reflect the official opinion of the European Union.

8. PARTICIPANTS (IN ALPHABETICAL ORDER BY COUNTRY)

Christoph Kefeder, AGES PharmMed, Austria
Maximilian Schreiner, Octapharma, Austria
Valerian Bunel, Scientific Institute of Public Health, Belgium
Valérie Lièvre, ANSM, France
Aline Laulan, LFB, France
Anna Hornik, Octapharma, France
Matthias Germer, Biotest Pharma GmbH, Germany
Daniela Karra, Paul Ehrlich Institut, Germany
Francesco Marino, Maria Wirz, Istituto Superiore di Sanità, Italy
Andrea Buzzigoli, Sossio Costanzo, Ilaria Rossi, Kedrion, Italy
Berber de Vries, RIVM, the Netherlands
Luis Meirinhos Soares, INFARMED, Portugal
Isabel Rodrigo, AEMPS, Spain
Nuria Hosta, Instituto Grifols SA, Spain
Stefan Spycher, Daniel Frattini, CSL Behring AG, Switzerland
Katja Boegli, Swissmedic, Switzerland
Carl Dolman, NIBSC, UK

9. ABBREVIATIONS

ACA: anticomplementary activity; BRP: Biological Reference Preparation; BSP: Biological Standardisation Programme; cBRP: candidate Biological Reference Preparation; EDQM: European Directorate for the Quality of Medicines & HealthCare; HBsAg: hepatitis B surface antigen; HCV: hepatitis C virus; HIV: human immunodeficiency virus; IFc: index of Fc function; IgG: immunoglobulin G; Lab: laboratory; OMCL: Official Medicines Control Laboratory; PEI: Paul-Ehrlich Institut; Ph. Eur.: European Pharmacopoeia; RR: Relative Retention; RSD: Relative Standard Deviation; SD: Standard Deviation; SEC: Size-Exclusion Chromatography; SRH: Single Radial Haemolysis; WHO: World Health Organization.

10. REFERENCES

[1] Buchacher A, Schluga P, Müllner J *et al.* Anticomplementary activity of IVIG concentrates – important assay parameters and impact of IgG polymers. *Vox Sang* 2010; 98 (3): 209-18.

[2] Human normal immunoglobulin for intramuscular administration, monograph 0338. Ph. Eur. 9th Edition. Strasbourg, France: Council of Europe; 2016.

[3] Human normal immunoglobulin for intravenous administration, monograph 0918. Ph. Eur. 9th Edition. Strasbourg, France: Council of Europe; 2016.

[4] Human immunoglobulin for subcutaneous administration, monograph 2788. Ph. Eur. 9th Edition. Strasbourg, France: Council of Europe; 2016.

[5] Anti-T lymphocyte immunoglobulin for human use, animal, monograph 1928. Ph. Eur. 9th Edition. Strasbourg, France: Council of Europe; 2016.

[6] Sandberg E, Costanzo A, Daas A *et al.* Calibration of the human immunoglobulin BRPs for ACA and molecular size (batch 1) and for Fc function and molecular size (batches 1 & 2). *Pharmeur Bio Sci Notes* 2012: 1-15.

[7] Test for Fc function of immunoglobulin, general chapter 2.7.9. Ph. Eur. 9th Edition. Strasbourg, France: Council of Europe; 2016.

[8] Test for anticomplementary activity of immunoglobulin, general chapter 2.6.17. Ph. Eur. 9th Edition. Strasbourg, France: Council of Europe; 2016.

APPENDIX

Overview of Fc function results from Day 1 (participants' calculations)

Lab	Assay	Vial	Mean corrected slope				Index of Fc function	
			Control	Sample A	Sample B	Sample F	A versus F	B versus F
2	1	1	0,001	0,089	0,090	0,094	91,7	92,4
		2	0,001	0,090	0,088	0,094	92,6	90,4
	2	1	0,003	0,087	0,088	0,087	94,9	95,7
		2	0,003	0,087	0,087	0,087	95,6	95,4
	3	1	0,005	0,088	0,089	0,097	93,9	95,4
		2	0,005	0,091	0,088	0,097	97,2	94,3
3	1	1	0,053	0,219	0,214	0,213	104,0	100,6
		2	0,053	0,211	0,219	0,213	98,7	103,9
	2	1	0,050	0,226	0,238	0,205	113,5	121,2
		2	0,050	0,232	0,237	0,205	117,0	120,1
	3	1	0,099	0,203	0,216	0,206	97,4	109,6
		2	0,099	0,213	0,222	0,206	107,0	115,3
8	1	1	0,018	0,393	0,377	0,368	107,0	103,0
		2	0,018	0,390	0,373	0,368	106,0	101,0
	2	1	0,021	0,426	0,409	0,399	107,0	103,0
		2	0,021	0,415	0,409	0,399	104,0	103,0
	3	1	0,014	0,349	0,352	0,340	103,0	104,0
		2	0,014	0,349	0,346	0,340	103,0	102,0
13	1	1	0,038	0,160	n.t.	0,159	100,8	n.t.
			0,013	n.t.	0,114	0,154	n.t.	70,8
		2	0,042	0,157	n.t.	0,178	83,8	n.t.
			0,043	n.t.	0,183	0,203	n.t.	87,5
	2	1	0,030	0,164	n.t.	0,157	105,9	n.t.
			0,030	n.t.	0,163	0,172	n.t.	93,7
		2	0,040	0,184	n.t.	0,214	96,5	n.t.
			0,036	n.t.	0,190	0,218	n.t.	84,3
	3	1	0,038	0,160	n.t.	0,158	101,5	n.t.
			0,013	n.t.	0,114	0,154	n.t.	71,6
		2	0,033	0,168	n.t.	0,209	76,8	n.t.
			0,031	n.t.	0,164	0,215	n.t.	72,9
14	1	1	-0,031	-0,155	-0,147	-0,159	96,3	90,4
		2	-0,031	-0,160	-0,154	-0,159	100,4	96,0
	2	1	0,029	-0,127	-0,124	-0,144	90,1	88,4
		2	0,029	-0,124	-0,142	-0,144	88,0	98,8
	3	1	0,016	-0,107	-0,109	-0,141	78,3	79,7
		2	0,016	-0,101	-0,115	-0,141	75,0	83,9
17	1	1	-0,040	-0,350	-0,330	-0,310	113,0	108,0
		2	-0,040	-0,340	-0,330	-0,310	110,0	106,0
	2	1	-0,070	-0,380	-0,370	-0,370	104,0	101,0
		2	-0,070	-0,370	-0,380	-0,370	101,0	105,0
	3	1	-0,070	-0,360	-0,380	-0,350	106,0	110,0
		2	-0,070	-0,360	-0,380	-0,350	104,0	114,0
						N	6	6
						Mean	99,0	97,6
						SD	7,9	11,9

Lab: laboratory; n.t.: not tested; SD: standard deviation; N: number of laboratories.

Overview of ACA results from Day 1 (participants' calculations)

			Compl. Control		Sample E						Sample C				Sample D			
			Mean activity		Negative		Positive				Negative		Positive		Negative		Positive	
Lab	Assay	Repl.	(CH50/mL)	Slope	% Act	Slope	% Act	Slope	Vial	Repl.	% Act	Slope	% Act	Slope	% Act	Slope	% Act	Slope
2	1	1	93,6	0,250	18,5%	0,260	76,3%	0,250	1	1	24,6%	0,310	91,3%	0,310	25,7%	0,260	91,3%	0,290
		2	93,4	0,240	18,3%	0,280	76%	0,240		2	25,6%	0,310	91,2%	0,300	25,6%	0,250	91,4%	0,280
	2	1	91,1	0,200	22,3%	0,200	73,5%	0,230	1	1	27,3%	0,190	Inv	Inv	28%	0,200	Inv	Inv
		2	90,1	0,190	21,6%	0,190	73,8%	0,230		2	26%	0,190	Inv	Inv	25,4%	0,220	Inv	Inv
	3*	1	Inv	0,290	Inv	Inv	Inv	Inv	1	1	Inv	Inv	Inv	Inv	Inv	Inv	Inv	Inv
		2	Inv	***0,410***	Inv	Inv	Inv	Inv		2	Inv	Inv	Inv	Inv	Inv	Inv	Inv	Inv
	4	1	101,0	0,200	23,2%	0,210	Inv	0,210	1	1	28,9%	0,230	Inv	Inv	27%	0,200	Inv	Inv
		2	101,4	0,180	22,2%	0,210	69,9%	0,260		2	27,4%	0,220	Inv	Inv	27,9%	0,220	Inv	Inv
3	1	1	94,0	0,190	16%	0,200	82%	0,250	1	1	19%	0,200	94%	0,290	20%	0,200	95%	0,310
										2	19%	0,200	94%	0,290	20%	0,200	95%	0,310
		2	93,9	0,190	16%	0,200	82%	0,250	2	1	20%	0,210	94%	0,290	19%	0,210	94%	0,290
										2	20%	0,210	94%	0,290	19%	0,210	94%	0,290
	2	1	92,4	0,200	18%	0,220	78%	0,280	1	1	19%	0,220	94%	0,300	19%	0,210	95%	0,320
										2	19%	0,220	94%	0,300	19%	0,210	95%	0,310
		2	92,4	0,200	18%	0,220	78%	0,280	2	1	21%	0,230	94%	0,300	21%	0,220	94%	0,300
										2	21%	0,230	94%	0,300	21%	0,220	94%	0,300
	3	1	91,5	0,210	16%	0,210	80%	0,250	1	1	17%	0,210	94%	0,290	17%	0,200	94%	0,310
										2	17%	0,210	94%	0,290	17%	0,200	94%	0,310
		2	91,4	0,210	16%	0,210	80%	0,250	2	1	16%	0,220	94%	0,300	18%	0,200	94%	0,300
		2								2	16%	0,220	94%	0,290	18%	0,200	94%	0,300
5	1	1	99,0	0,163	13%	0,177	76%	0,232	1	1	20%	0,171	87%	0,249	19%	0,170	81%	0,237
								0,231		2	20%	0,171	87%	0,260	18%	0,168	85%	0,248
		2	99,0	0,163	12%	0,173	74%	0,231	2	1	20%	0,172	86%	0,257	18%	0,172	89%	0,285
		2								2	20%	0,172	86%	0,249	17%	0,168	89%	0,273
	2	1	104,0	0,158	14%	0,164	73%	0,204	1	1	20%	0,173	74%	0,214	19%	0,173	85%	0,264
										2	20%	0,166	77%	0,255	19%	0,170	79%	0,251
		2	104,0	0,157	11%	0,183	76%	0,239	2	1	20%	0,172	82%	0,226	19%	0,180	82%	0,249
		2								2	20%	0,175	84%	0,250	19%	0,179	89%	0,287
	3	1	103,0	0,160	12%	0,199	76%	0,247	1	1	19%	0,174	92%	0,331	19%	0,173	92%	0,338
										2	20%	0,171	91%	0,312	19%	0,167	92%	0,343
		2	101,0	0,165	11%	0,199	75%	0,241	2	1	18%	0,185	91%	0,343	16%	0,209	91%	0,361
		2								2	18%	0,180	89%	0,320	15%	0,218	91%	0,359
13	1a	1	108,0	0,235	29,2%	0,245	69,2%	0,254	1	1	32,6%	0,263	91,4%	0,397	29,3%	0,238	91%	0,384
		2		0,247	20,9%	0,244	74,4%	0,267		2	31,9%	0,248	97,1%	***0,578***	33,4%	0,239	86,3%	***0,651***
	1b	1	117,7	0,199	26,6%	0,210	61,6%	0,208	2	1	41,2%	0,230	90%	0,322	40,3%	0,229	93%	0,367
		2		0,201	35,1%	0,217	66,7%	0,244		2	41,4%	0,230	90,5%	0,356	44,8%	0,239	91,3%	0,343
	2a	1	80,3	0,373	25,1%	0,379	70,5%	0,377	1	1	28,1%	0,383	86,3%	0,361	27%	0,385	88,5%	0,356
		2		0,392	24,9%	0,390	70,4%	0,374		2	28,9%	***0,400***	93,9%	0,399	28,8%	0,398	83,2%	0,350
	2b	1	80,6	0,394	25,6%	0,380	64%	0,379	2	1	33,1%	0,382	87,6%	0,357	29,7%	0,344	88,4%	0,331
		2		0,361	27,9%	0,386	72,4%	0,382		2	36,8%	0,383	90,2%	0,345	34%	0,348	88,9%	0,341
	3	1	89,1	0,318	18,3%	0,328	68,9%	0,343	1	1	20,2%	0,339	89,7%	0,334	20,3%	0,343	90,5%	0,345
										2	24,8%	0,339	90,2%	0,334	22,3%	0,350	90,1%	0,323
		2		0,311	20,1%	0,344	70,7%	0,332	2	1	21,5%	0,350	81,8%	0,356	27,1%	0,352	85,8%	0,343
		2								2	24,9%	0,344	87,5%	0,340	27,4%	0,346	88,9%	0,329
14	1	1	*87,1*	*0,200*	*12%*	*0,200*	50%	*0,240*	1	1	*19%*	*0,200*	*84%*	*0,260*	*18%*	*0,210*	*84%*	*0,270*
										2	*17%*	*0,200*	*83%*	*0,270*	*18%*	*0,210*	*84%*	*0,260*
		2	*89,7*	*0,190*	*14%*	*0,190*	52%	*0,240*	2	1	*19%*	*0,200*	*83%*	*0,270*	*19%*	*0,210*	*84%*	*0,270*
		2								2	*19%*	*0,210*	*83%*	*0,270*	*18%*	*0,200*	*84%*	*0,270*
	2	1	*83,0*	*0,200*	*18%*	*0,220*	55%	*0,240*	1	1	*24%*	*0,220*	*59%*	*0,230*	*23%*	*0,220*	*58%*	*0,230*
										2	*24%*	*0,220*	*59%*	*0,230*	*24%*	*0,220*	*58%*	*0,230*
		2	*82,1*	*0,200*	*22%*	*0,230*	55%	*0,240*	2	1	*23%*	*0,230*	*58%*	*0,230*	*23%*	*0,220*	*58%*	*0,230*
		2								2	*25%*	*0,220*	*58%*	*0,230*	*24%*	*0,220*	*58%*	*0,230*
	3	1	*92,2*	*0,200*	*20%*	*0,220*	49%	*0,250*	1	1	*24%*	*0,220*	*82%*	*0,260*	*24%*	*0,220*	*82%*	*0,250*
										2	*23%*	*0,220*	*81%*	*0,260*	*25%*	*0,220*	*80%*	*0,270*
		2	*91,3*	*0,210*	*22%*	*0,220*	48%	*0,210*	2	1	*24%*	*0,220*	*82%*	*0,260*	*23%*	*0,220*	*82%*	*0,260*
		2								2	*25%*	*0,220*	*81%*	*0,260*	*22%*	*0,220*	*80%*	*0,200*
16	1	1							1	1	*30%*	*0,250*	*154%*	*0,230*	*28%*	*0,250*	*146%*	*0,200*
		2	*103,0*	*0,240*	*10%*	*0,240*	126%	*0,230*		2								
		2							2	1	*30%*	*0,240*	*146%*	*0,220*	*28%*	*0,250*	*150%*	*0,190*
										2								
17	1	1	91,3	0,350	15,5%	0,360	67,7%	0,340	1	1	18,8%	0,370	94,3%	***0,430***	17,7%	0,360	93,5%	0,390
		2								2	21,2%	0,360	94,1%	***0,440***	17,4%	0,360	94,1%	***0,440***
		2			17%	0,370	67,8%	0,330	2	1	18,3%	0,360	94,2%	***0,430***	16%	0,360	94,3%	***0,450***
		2								2	18,1%	0,360	93,7%	***0,450***	17,3%	0,350	94,2%	***0,430***
	2	1	87,3	0,370	14,2%	0,390	60,8%	0,340	1	1	17,2%	0,380	92%	***0,420***	14,9%	0,380	93,2%	***0,450***
										2	18,5%	0,390	91,7%	***0,450***	14,6%	0,370	92,1%	***0,410***
		2			13,7%	0,380	62,2%	0,330	2	1	16,8%	0,380	90,1%	***0,430***	14,1%	0,380	90,6%	***0,420***
		2								2	15,9%	0,380	91,5%	***0,430***	13,9%	0,380	89,7%	***0,440***
	3	1	98,3	0,330	14,7%	0,360	69,9%	0,310	1	1	17,6%	0,350	91,3%	0,390	15,6%	0,330	90,5%	0,390
										2	16,3%	0,340	93,1%	0,380	16,8%	0,350	93,1%	0,350
		2			13,9%	0,340	70,2%	0,300	2	1	13,1%	0,330	93%	0,360	15,4%	0,330	93,5%	0,380
		2								2	13,4%	0,330	92,7%	0,370	14,1%	0,330	93,5%	0,380
Including all tests				N	7		7				7		7		7		7	
				Mean	16,9		77,4				23,5		96,8		22,8		96,7	
				SD	5,23		23,28				5,50		24,38		5,59		23,58	
Excluding invalid tests				N	5		5				5		5		5		5	
				Mean	18,0		72,8				22,5		90,5		21,9		90,9	
				SD	5,22		5,33				5,81		3,32		6,24		2,90	

Values causing the test to be invalid according to Ph. Eur. chapter 2.6.17 are printed on a dark background. Corresponding calculated results are indicated in italics. Shaded cells show valid slopes outside the preferred range (0.18-0.30). Slopes outside the 0.15-0.40 range are in bold italics.
Inv: absence of a straight line between 15% and 85% haemolysis.
Act: activity; Comp: complement; Lab: laboratory; N: number of laboratories, Repl: replicate; SD: standard deviation.
* This assay was considered invalid by the participant due to high haemolysis in some tubes.

Fc function results for the short-term stability assessment (participants' calculations)

Day 7							
Lab		Vial	Mean corrected slope				Index of Fc function	
			Control	Sample A	Sample B	Sample F	A versus F	B versus F
3		1	0,088	0,192	0,187	0,177	117,3	111,6
		2	0,088	0,180	0,192	0,177	104,0	116,6
8		1	0,017	0,371	0,386	0,350	106,0	111,0
		2	0,017	0,373	0,360	0,350	107,0	103,0
13	assay 1	1	0,043	0,203	n.t.	0,221	94,0	n.t.
			0,038	n.t.	0,221	0,229	n.t.	96,1
		2	0,047	0,208	n.t.	0,223	95,5	n.t.
			0,042	n.t.	0,221	0,220	n.t.	100,5
	assay 2	1	0,040	0,216	n.t.	0,213	101,9	n.t.
			0,039	n.t.	0,210	0,218	n.t.	95,9
		2	0,045	0,218	n.t.	0,222	97,5	n.t.
			0,042	n.t.	0,224	0,224	n.t.	100,0
	assay 3	1	0,040	0,211	n.t.	0,219	95,2	n.t.
			0,032	n.t.	0,213	0,218	n.t.	98,0
		2	0,030	0,187	n.t.	0,220	82,8	n.t.
			0,031	n.t.	0,200	0,211	n.t.	93,7
14		1	-0,024	-0,134	-0,116	-0,133	101,1	84,9
		2	-0,024	-0,124	-0,109	-0,133	91,8	78,2
17		1	-0,060	-0,360	-0,370	-0,350	103,0	104,0
		2	-0,060	-0,370	-0,370	-0,350	107,0	105,0
Including all tests						N	5	5
						Mean	102,6	100,9
						SD	6,9	12,4

Day 14							
Lab		Vial	Mean corrected slope				Index of Fc function	
			Control	Sample A	Sample B	Sample F	A versus F	B versus F
3		1	0,107	0,200	0,224	0,190	112,0	140,7
		2	0,107	0,205	0,226	0,190	117,9	142,7
8		1	0,022	0,371	0,374	0,371	100,0	101,0
		2	0,022	0,383	0,366	0,371	103,0	99,0
13	assay 1	1	0,038	0,142	n.t.	0,179	73,5	n.t.
			0,042	n.t.	0,146	0,186	n.t.	72,5
		2	0,036	0,127	n.t.	0,191	58,4	n.t.
			0,039	n.t.	0,176	0,193	n.t.	89,2
	assay 2	1	0,033	0,150	n.t.	0,186	77,6	n.t.
			0,031	n.t.	0,174	0,197	n.t.	85,6
		2	0,038	0,189	n.t.	0,199	93,8	n.t.
			0,027	n.t.	0,167	0,193	n.t.	84,5
	assay 3	1	0,034	0,171	n.t.	0,204	80,6	n.t.
			0,036	n.t.	0,184	0,202	n.t.	89,5
		2	0,034	0,159	n.t.	0,188	81,2	n.t.
			0,030	n.t.	0,147	0,163	n.t.	88,9
14		1	-0,024	-0,181	-0,152	-0,186	96,8	79,2
		2	-0,024	-0,162	-0,149	-0,186	84,9	76,9
17		1	-0,050	-0,290	-0,280	-0,300	96,0	95,0
		2	-0,050	-0,300	-0,280	-0,300	101,0	95,0
Including all tests						N	5	5
						Mean	96,7	100,0
						SD	13,8	24,8
Excluding invalid tests						N		4
						Mean		89,5
						SD		9,9

Lab: laboratory; N: number of laboratories; n.t.: not tested; SD: standard deviation. Invalid results are presented on a shaded background.

ACA results for the short-term stability assessment (participants' calculations)

Day 7

		Compl. Control			Sample E							Sample C				Sample D			
						Negative		Positive				Negative		Positive		Negative		Positive	
Lab	Assay	Repl.	Mean activity (CH50/mL)	Slope	Repl.	% Act	Slope	% Act	Slope	Vial	Repl.	% Act	Slope	% Act	Slope	% Act	Slope	% Act	Slope
3		1	96,4	0,200	1	19%	0,210	82%	0,250	1	1	20%	0,250	94%	0,31	20%	0,210	94%	0,31
										2	2	21%	0,230	95%	0,32	24%	0,230	94%	0,300
5		1	95,0	0,220	1	14%	0,320	75%	0,231	1	1	21%	0,263	91%	0,308	18%	0,224	90%	0,307
		2								2	2	23%	0,373	91%	0,315	19%	0,212	91%	0,312
13	1a	1	90,0	0,396	1	19,42%	0,355	73,5%	0,313	1	1	25,32%	0,3363	86,54%	0,3495	23,17%	0,3312	83,65%	0,3541
		2		0,378	2	24,46%	0,315	72%	0,340		2	28,84%	0,319	86,99%	0,3439	29,52%	0,336	85,49%	0,3544
	1b	1	104,3	0,250	1	26,57%	0,278	65,5%	0,278	1	1	29,41%	0,292	90,2%	0,3092	29,34%	0,283	85%	0,286
		2		0,242	2	27,81%	0,275	69,8%	0,283		2	37,87%	0,283	90,8%	0,3134	37,72%	0,287	89,5%	0,302
	2a	1	97,9	0,350	1	20,65%	0,343	51%	0,360	1	1	23,84%	0,3474	90,26%	0,3558	22,55%	0,3473	90,25%	0,340
		2		0,351	2	22,07%	0,343	66,4%	0,338		2	27,57%	0,348	89,82%	0,3476	27,39%	0,3452	90,7%	0,351
	2b	1	97,1	0,337	1	21,04%	0,344	74,9%	0,329	1	1	23,07%	0,3266	90,91%	0,3534	25,35%	0,3294	90,16%	0,3437
		2		0,334	2	21,74%	0,354	76,6%	0,333		2	26,55%	0,3307	91,71%	0,3538	28,96%	0,3617	88,09%	0,3432
	3	1	97,1	0,285	1	29,26%	0,293	68,8%	0,292	1	1	36,72%	0,3112	90,2%	0,299	n.t.	n.t.	89%	0,3031
											2	35,56%	0,3034	93,3%	0,3358	43,59%	0,3178	94,7%	0,3748
		2		0,279	2	26,89%	0,295	67,6%	0,303	2	1	n.t.	n.t.	94,2%	0,3414	24,54%	0,299	94,6%	0,3678
											2	n.t.	n.t.	96%	***0,413***	35,78%	0,3056	96,6%	***0,452***
14		1	83,1	0,190	1	19%	0,220	57%	0,250	1	1	25%	0,220	84%	0,260	25%	0,220	84%	0,270
		2	83,0	0,180						2	1	26%	0,220	84%	0,260	26%	0,220	85%	0,280
17		1	101,4	0,310	1	13,2%	0,330	69,9%	0,290	1	1	18,7%	0,34	89,2%	0,38	15%	0,320	91,6%	0,400
		2								2	1	16,5%	0,33	85,7%	0,37	15,1%	0,330	87,5%	0,380
Including all tests					N	5		5				5		5		5		5	
					Mean	17,8		70,5				20,1		78,1		19,5		78,1	
					SD	4,38		9,20				6,54		19,23		7,24		18,41	
Excluding invalid tests					N	4		4				4		4		4		4	
					Mean	17,6		74,4				21,1		83,6		20,3		83,3	
					SD	5,16		5,57				7,53		16,94		8,49		15,88	

Day 14

		Compl. Control			Sample E							Sample C				Sample D			
						Negative		Positive				Negative		Positive		Negative		Positive	
Lab	Assay	Repl.	Mean activity (CH50/mL)	Slope	Repl.	% Act	Slope	% Act	Slope	Vial	Repl.	% Act	Slope	% Act	Slope	% Act	Slope	% Act	Slope
3		1	91,3	0,280	1	22%	0,390	81%	0,290	1	1	17%	0,240	93%	0,25	20%	0,190	94%	0,27
										2	2	17%	0,250	93%	0,26	19%	0,230	94%	0,27
5		1	100,0	0,174	1	21%	0,185	74%	0,372	1	1	28%	0,205	77%	0,241	24%	0,210	71%	0,222
		2								2	2	25%	0,198	77%	0,262	25%	0,203	69%	0,272
13	1a	1	94,4	0,231	1	30,75%	0,248	72,3%	0,238	1	1	30,63%	0,254	92,8%	0,310	34,9%	0,268	91,6%	0,286
											2	35,37%	0,254	93,9%	0,3229	40,12%	0,264	90,6%	0,285
	1b	2		0,229	2	28,97%	0,246	65,6%	0,249	2	1	39,2%	0,265	92%	0,300	34,54%	0,251	91,9%	0,290
											2	42,25%	0,276	92,5%	0,278	33,54%	0,256	93,5%	0,3043
	2a	1	99,8	0,261	1	24,06%	0,286	65,6%	0,285	1	1	36,65%	0,293	92,9%	0,331	29,57%	0,294	91,3%	0,3135
											2	38,14%	0,299	93,8%	0,3413	36,74%	0,289	94,1%	0,3624
	2b	2		0,255	2	26,29%	0,281	77,3%	0,298	2	1	28,51%	0,3043	93,9%	0,3388	29,73%	0,292	91,4%	0,3143
											2	32,93%	0,297	92,5%	0,3313	33,04%	0,292	93,7%	0,3334
	3	1	116,5	0,230	1	26,25%	0,222	73%	0,238	1	1	28,81%	0,240	84,4%	0,227	29,9%	0,239	91,6%	0,272
											2	34,26%	0,245	79,4%	0,220	33,18%	0,240	92,7%	0,292
		2		0,211	2	25,9%	0,223	78%	0,252	2	1	35,52%	0,263	80,8%	0,249	28,12%	0,245	91,6%	0,275
											2	35,88%	0,257	92,1%	0,297	31,16%	0,234	92,7%	0,298
14		1	79,4	0,190	1	13%	0,200	48%	0,220	1	1	23%	0,200	82%	0,280	22%	0,200	82%	0,260
		2	82,1	0,180						2	1	23%	0,200	83%	0,320	23%	0,200	82%	0,260
17		1	99,1	0,320	1	15,9%	0,340	66,9%	0,290	1	1	22,3%	0,320	92,6%	0,370	20,8%	0,320	94,1%	0,350
		2								2	1	22,1%	0,320	91,7%	0,360	22,5%	0,340	93,8%	0,380
Including all tests					N	5		5				5		5		5		5	
					Mean	19,8		68,4				21,7		75,3		21,3		74,7	
					SD	5,48		12,46				8,75		16,88		7,65		17,67	
Excluding invalid tests					N	4		4				4		4		4		4	
					Mean	21,5		73,5				23,3		80,4		22,8		79,7	
					SD	4,56		5,84				9,23		14,42		7,86		15,78	

Values causing the test to be invalid according to Ph. Eur. monograph requirements are printed on a dark background. Valid slopes outside the preferred range of 0.18-0.30 are printed on a shaded background. Slopes outside the 0.15-0.40 range are in bold italics.
Act: activity; Comp: complement; Lab: laboratory; n.t.: not tested; Repl: replicate; SD: standard deviation.

Summary of peak areas observed by SEC on Day 1 (as reported by participants)

Lab	Assay	Peak area monomer + dimer (%)					Peak area aggregates/polymers (%)				
		Sample A		Sample B			Sample A		Sample B		
		Vial 1	Vial 2	Vial 1	Vial 2	Sample E	Vial 1	Vial 2	Vial 1	Vial 2	Sample E
1	1	99,9	99,9	99,9	99,9	99,4	0,1	0,1	0,1	0,1	0,6
	2	99,9	99,9	99,9	99,9	99,4	0,1	0,1	0,1	0,1	0,6
2	1	99,0	99,0	99,0	99,1	99,1	0,4	0,4	0,3	0,3	0,9
	2	99,0	99,0	99,0	99,0	99,1	0,4	0,4	0,3	0,4	0,9
3a	1	98,9	98,9	98,9	99,0	99,1	0,2	0,3	0,2	0,2	0,5
	2	98,9	98,8	98,9	98,9	99,0	0,2	0,2	0,3	0,2	0,5
3b	1	99,7	99,7	99,7	99,7	99,4	0,3	0,3	0,3	0,3	0,5
	2	99,7	99,7	99,7	99,7	99,3	0,3	0,3	0,3	0,3	0,7
4 version 1	1	99,1	99,0	99,1	99,0	99,3	0,2	0,2	0,2	0,2	0,7
	2	99,1	99,0	99,1	99,1	99,3	0,2	0,2	0,2	0,2	0,8
4 version 2	1	99,1	99,0	99,1	99,1	99,2	0,2	0,2	0,2	0,2	0,8
	2	99,1	99,0	99,1	99,1	99,2	0,2	0,2	0,2	0,2	0,8
4 version 3	1	99,1	99,0	99,1	99,2	99,3	0,2	0,2	0,2	0,1	0,8
	2	99,1	99,1	99,2	99,2	99,3	0,1	0,1	0,1	0,1	0,7
5	1	99,0	99,0	99,1	99,0	99,7	0,2	0,2	0,2	0,2	0,3
	2	99,1	98,9	98,9	99,0	99,7	0,2	0,2	0,2	0,2	0,3
6	1	98,9	98,9	99,0	99,0	98,1	0,2	0,2	0,2	0,2	1,9
	2	98,9	98,9	99,0	99,0	99,5	0,2	0,2	0,2	0,2	0,5
7	1	100,0	99,9	99,8	99,9	98,9	0,0	0,1	0,2	0,1	1,1
	2	99,8	99,7	99,7	99,8	99,6	0,2	0,3	0,3	0,3	0,4
8	1	99,1	99,1	99,0	99,2	99,5	<0.1	<0.1	<0.1	<0.1	0,4
	2	99,1	99,0	99,2	99,2	99,4	<0.1	<0.1	<0.1	<0.1	0,5
9	1	99,5	99,5	99,5	99,5	99,7	n.r.	n.r.	n.r.	n.r.	0,3
	2	99,5	99,5	99,5	99,6	99,7	n.r.	n.r.	n.r.	n.r.	0,3
10	1	98,0	98,1	98,1	98,1	97,9	0,2	0,2	0,2	0,3	0,5
	2	98,1	98,2	98,2	98,2	98,1	0,2	0,2	0,2	0,2	0,5
11	1	100,0	100,0	100,0	100,0	100,0					
	2	100,0	100,0	100,0	100,0	100,0					
12	1	99,9	99,9	99,9	99,9	99,6	0,1	0,1	0,1	0,1	0,4
	2	99,9	99,9	99,9	99,9	99,6	0,1	0,1	0,1	0,1	0,4
13	1	99,0	99,0	99,0	98,8	99,8	n.r.	n.r.	n.r.	n.r.	n.r.
	2	98,7	98,7	98,7	98,9	99,9	n.r.	n.r.	n.r.	n.r.	n.r.
14	1	98,5	98,4	98,5	98,5	97,9	0.19*	0.23*	0.22*	0.19*	0,5
	2	98,4	98,4	98,5	98,4	97,9	0.18*	0.19*	0.16*	0.16*	0,5
15	1	99,8	99,8	99,8	99,8	100,0	0,2	0,2	0,2	0,2	none
	2	99,8	99,8	99,8	99,8	99,6	0,2	0,2	0,2	0,2	0,4
16	1	98,9	98,9	98,9	98,9	98,4	0,2	0,2	0,2	0,2	0,6
	2	98,9	98,8	98,9	98,9	99,2	0,2	0,2	0,2	0,2	0,6
N		17		17		17	14		14		15
Mean		99,2		99,2		99,2	0,2		0,2		0,6
SD		0,56		0,55		0,60	0,07		0,07		0,24
RSD		0,57		0,55		0,61	35,55		34,10		41,96

N: number of laboratories; n.r. not reported; RSD: relative standard deviation; SD: standard deviation;
Three different methods were used by laboratory 4. See text for details.
< x: below validated quantification limit.
* The laboratory reported that the validated quantification limit for aggregates/polymers was 0.4 %.

SEC data for the short-term stability assessment (as reported by participants)

Day 7											
		Peak area monomer + dimer (%)					Peak area aggregates/polymers (%)				
		Sample A		Sample B			Sample A		Sample B		
Lab	Assay	Vial 1	Vial 2	Vial 1	Vial 2	Sample E	Vial 1	Vial 2	Vial 1	Vial 2	Sample E
1		99,9	99,9	99,9	99,9	99,4	0,1	0,1	0,1	0,1	0,6
3a		99,0	98,9	98,9	99,0	99,0	0,2	0,2	0,2	0,2	0,5
3b		99,7	99,7	99,7	99,7	99,3	0,3	0,3	0,3	0,3	0,7
4	version 1	99,0	99,0	99,1	99,1	99,3	0,2	0,2	0,2	0,2	0,7
	version 2	99,1	99,1	99,1	99,1	99,2	0,2	0,2	0,2	0,2	0,8
	version 3	99,1	99,1	99,2	99,1	99,3	0,1	0,1	0,1	0,1	0,7
5		98,7	98,7	98,9	98,8	99,7	0,2	0,2	0,2	0,2	0,3
6		98,9	98,9	99,0	99,0	n.r.	0,2	0,2	0,2	0,2	n.r.
7		99,9	99,8	99,8	99,8	99,8	0,2	0,2	0,2	0,2	0,2
8		98,9	98,9	98,9	98,9	99,3	0,2	0,2	<0.1	<0.1	0,6
9		99,5	99,5	99,5	99,5	99,7	n.r.	n.r.	n.r.	n.r.	0,3
10		98,1	98,1	98,1	98,0	97,9	0,2	0,2	0,2	0,3	0,5
11		100,0	100,0	100,0	100,0	100,0					
12		99,9	99,9	99,9	99,9	99,4	0,1	0,1	0,1	0,1	0,6
13		98,9	98,7	98,9	98,9	99,9	n.r.	n.r.	n.r.	n.r.	n.r.
14		98,5	98,5	98,5	98,5	97,9	0.19*	0.18*	0.18*	0.18*	0,5
15		99,8	99,8	99,8	99,8	99,7	0,2	0,2	0,2	0,2	0,3
16	1	98,9	98,8	98,8	98,9	96,2	0,2	0,2	0,2	0,2	0,6
	2	98,9	98,8	98,9	98,9	99,2	0,3	0,3	0,2	0,2	0,7
N		16		16		15	13		13		13
Mean		99,2		99,2		99,2	0,2		0,2		0,5
SD		0,58		0,56		0,76	0,05		0,06		0,16
RSD		0,59		0,57		0,77	26,41		30,54		32,90

Day 14											
		Peak area monomer + dimer (%)					Peak area aggregates/polymers (%)				
		Sample A		Sample B			Sample A		Sample B		
Lab	Assay	Vial 1	Vial 2	Vial 1	Vial 2	Sample E	Vial 1	Vial 2	Vial 1	Vial 2	Sample E
1		99,9	99,9	99,9	99,9	99,4	0,1	0,1	0,1	0,1	0,6
3a		98,8	98,9	98,9	98,8	99,0	0,2	0,2	0,2	0,2	0,5
3b		99,7	99,7	99,7	99,7	99,4	0,3	0,3	0,3	0,3	0,6
4	version 1	99,0	99,1	99,0	99,1	99,3	0,2	0,2	0,2	0,2	0,7
	version 2	99,0	99,0	99,1	99,1	99,2	0,2	0,2	0,2	0,2	0,8
	version 3	99,1	99,1	99,1	99,1	99,3	0,2	0,2	0,2	0,2	0,8
5		99,1	99,1	99,1	99,2	99,7	0,1	0,1	0,1	0,1	0,3
6		98,9	98,9	98,9	98,9	98,2	0,2	0,2	0,2	0,2	1,8
7		99,8	99,8	99,8	99,8	99,7	0,2	0,2	0,2	0,3	0,3
8		98,9	98,9	98,9	99,0	99,4	<0.1	<0.1	0,2	<0.1	0,5
9		99,5	99,5	99,5	99,5	99,7	n.a.	n.a.	n.a.	n.a.	0,3
10		98,1	98,1	98,2	98,1	97,9	0,2	0,2	0,2	0,2	0,5
11		100,0	100,0	100,0	100,0	100,0					
12		99,9	99,9	99,9	99,9	99,5	0,1	0,1	0,1	0,1	0,5
13		99,1	99,1	99,2	99,2	99,7	0,1	0,1	0,1	0,1	0,5
14		98,5	98,4	98,5	98,5	97,9	0.17*	0.19*	0.18*	0.17*	0,5
15		99,9	99,9	99,9	99,9	99,8	0,2	0,1	0,1	0,1	0,2
16	1	98,8	98,8	n.r.	n.r.	99,3	0,2	0,3	n.r	n.r.	0,6
	2	98,8	98,8	98,8	98,8	99,4	0,2	0,2	0,3	0,2	0,6
N		16		16		16	14		14		15
Mean		99,2		99,3		99,2	0,2		0,2		0,6
SD		0,56		0,55		0,66	0,06		0,07		0,38
RSD		0,57		0,55		0,67	37,18		38,52		68,37

n.r. not reported; N: number of laboratories; SD: standard deviation; RSD: relative standard deviation.
Three different methods were used by laboratory 4. See text for details.
< x: below validated quantification limit.
* The laboratory reported that the validated quantification limit for aggregates/polymers was 0.4 %.

Findings on the heavy metal content in herbal drugs and essential oils – an update

H. Albert[1], B. Klier[2], M. Knödler[3], B. Steinhoff[4]

ABSTRACT

In this contribution, data for 7 elemental impurities originating from quality control analysis of manufacturers of herbal products is evaluated in light of the current requirements of the European Pharmacopoeia (Ph. Eur.) and the European legislative framework. The data shows that the Ph. Eur. limits set for cadmium, lead and mercury in herbal drugs are in principle still appropriate. The probability of herbal drugs exceeding the limits for arsenic, cobalt, nickel and vanadium (based on the ICH Q3D guideline for elemental impurities) appears to be very low, and consequently, it is proposed that general limits for these elements in herbal drugs in the Ph. Eur. are not required. For essential oils, there does not appear to be a risk of heavy metal contamination and a general test on heavy metals is not considered necessary.

KEYWORDS

Heavy metals, lead, cadmium, mercury, arsenic, cobalt, nickel, vanadium, herbal drugs, essential oils, elemental impurities.

1. INTRODUCTION

For the past 20 years, several German companies have collected and evaluated data on heavy metals from their daily practices. In 1998 and 2009, reports were published [1, 2] and submitted to the European Pharmacopoeia (Ph. Eur.) for discussion. The general monograph *Herbal drugs (1433)* [3] was subsequently revised and published in Ph. Eur. 6.8, and included limits for cadmium, lead and mercury. A new evaluation of data originating from quality control analysis by manufacturers of herbal products between 2008 and 2015 is now presented. In addition to cadmium, lead and mercury, data is also included for arsenic, cobalt, nickel and vanadium. Compliance with existing limits, i.e. as laid down in the Ph. Eur., is discussed, while also taking into consideration the revision of the Ph. Eur. general monograph *Essential oils (2098)* [4] and the new ICH Q3D guideline on elemental impurities [5].

2. REGULATORY FRAMEWORK

Heavy metals, in particular arsenic (As), cadmium (Cd), lead (Pb) and mercury (Hg), pose a hazard to human and animal health, and consequently, their presence in plants used for medicinal purposes or for consumption is limited within the regulatory framework for medicinal products and foodstuffs.

The Ph. Eur. general monograph *Herbal drugs (1433)* [3] contains the following limits for specific heavy metals: cadmium 1.0 ppm, lead 5.0 ppm, mercury 0.1 ppm. They apply ‘unless

1 H. Albert, SALUS Haus GmbH & Co. KG, Bruckmühl, Germany.
2 B. Klier, PhytoLab GmbH & Co. KG, Vestenbergsgreuth, Germany.
3 M. Knödler, WALA Heilmittel GmbH, Bad Boll/Eckwälden, Germany.
4 B. Steinhoff (corresponding author’s email: steinhoff@bah-bonn.de), Bundesverband der Arzneimittel-Hersteller e.V. (BAH), Ubierstr. 71-73, D-53173 Bonn, Germany.

otherwise stated in an individual monograph or unless otherwise justified and authorised'. Furthermore, the monograph states 'where necessary, limits for other heavy metals may be required'.

Taking into account a naturally occurring higher content of specific heavy metals in certain herbal drugs, several special cases with higher authorised limits for cadmium and lead, as well as a specific limit for arsenic, have been included in individual monographs since the publication of Ph. Eur. 6.8. These cases are shown in Table 1.

Table 1 – *Limits for arsenic, cadmium and lead in individual Ph. Eur. monographs*

Heavy metal	Herbal drug	Monograph limit (ppm)
As	Kelp [6]	90
Cd	Kelp [6]	4
Cd	Fumitory [7]	1.5
Cd	Tormentil [8]	2.0
Cd	Willow bark [9]	2.0
Pb	Iceland moss [10]	10.0
Pb	Nettle root [11]	7.0

The requirements with regard to heavy metal limits in the Ph. Eur. general monograph *Herbal drugs for homoeopathic preparations (2045)* [12] are the same as those for *Herbal drugs (1433)* [3], including reference to the potential need for additional limits for other heavy metals, e.g. arsenic or nickel.

The Ph. Eur. general monograph *Herbal drug extracts (0765)* [13] states that 'where applicable, as a result of analysis of the herbal drug used for production and in view of the production process', additional tests for the extracts, e.g. heavy metals, may be necessary, with the same limits as those for Herbal drugs (1433) [3] 'unless otherwise stated in an individual extract monograph or unless otherwise justified and authorised'. It is also stated that 'where justified, herbal drugs used for the production of extracts may exceed the limits for heavy metals specified in the monograph *Herbal drugs (1433)* provided that the resulting extract satisfies the requirements for heavy metals' [13].

A revision of the Ph. Eur. general monograph *Essential oils (2098)* [4] is under preparation, and it is still open as to whether a reference to a test for heavy metals is necessary.

Ph. Eur. general chapter 2.4.27. *Heavy metals in herbal drugs and herbal drug preparations* [14] describes a method for determination of heavy metals which covers lead, arsenic, cadmium, copper, mercury and nickel. It is also noted that chapter 2.8.N12 of the German Pharmacopoeia (DAB) [15] contains a limit test for heavy metals in essential oils using a lead solution as a reference solution.

According to the ICH Q3D Guideline for elemental impurities [5], limits for elemental impurities have to be considered as part of a risk assessment in the final medicinal product. Herbal products are formally excluded from the scope of the guideline; however, it is up to the manufacturers, as part of the overall risk assessment of their products, to decide in which cases testing for specific elemental impurities, e.g. Class 1 (arsenic, cadmium, lead, mercury) and Class 2A (cobalt, nickel, vanadium) for oral preparations might be required. The ICH Q3D limits relating to an oral administration of 10 g of the medicinal product (Option 1) are as follows: arsenic 1.5 ppm, cadmium 0.5 ppm, lead 0.5 ppm, mercury 3 ppm, cobalt 5 ppm, nickel 20 ppm and vanadium 10 ppm.

Apart from the legal framework applicable to medicinal products, Commission Regulation (EC) No. 1881/2006 [16] contains the following limits for food supplements: lead 3.0 ppm, cadmium 1.0 ppm, mercury 0.10 ppm and a specific limit of 3.0 ppm for cadmium in seaweed products. The annexes of Regulation (EC) 396/2005 [17] include a maximum residue limit for the sum of mercury compounds of 0.02 ppm in herbal infusions, though a lower limit (0.01 ppm) for herbal infusions has recently been proposed [18].

In 2007, an overview of maximum values for toxic metals set by countries in different regions of the world was published by the World Health Organization (WHO), where a limit of 10 ppm for lead and 0.3 ppm for cadmium in herbal medicines was proposed [19].

3. INDUSTRY INITIATIVES

For more than 18 years, the German Medicines Manufacturers' Association (BAH) working group on contaminants has maintained a large database on heavy metals that includes data from several companies and provides a detailed and extensive overview of the heavy metal content of herbal drugs and essential oils. Evaluation of the database can illustrate the heavy metal content for each individual herbal drug or essential oil, as well as the occurrence of a particular metal in different herbal drugs or essential oils. In addition, various percentiles, e.g. the 90th percentiles [20], can be calculated and the results assessed according to different legal frameworks. Such data collections have also been used to provide health authorities and for example, the Ph. Eur., with current information on the actual occurrence of heavy metals in plants used for medicinal or food purposes.

The authors of this publication, on behalf of the BAH working group on contaminants, hereby present an updated evaluation of the data from daily quality control in order to discuss compliance with existing limits laid down in the Ph. Eur. [3] or, in the case of certain elemental impurities, with the limits of ICH Q3D [5].

4. MATERIALS AND METHODS

The evaluation of data was performed using results from testing carried out over a period of 8 years between 1 January 2008 and 31 December 2015. The total number of samples was 18 401 (628 products), of which 5 304 (204 products) came from organic production. For cadmium, 17 071 sets of data were evaluated, for lead 16 954, for mercury 13 277, for nickel 4 336, for vanadium 4 051, for cobalt 4 022 and for arsenic 2 028. Determination of these heavy metals was performed using validated methods relying on analytical techniques such as atomic absorption spectrometry (AAS), inductively coupled plasma-mass spectrometry (ICP-MS) or inductively coupled plasma-optical emission spectrometry (ICP-OES), in accordance with Ph. Eur. general chapter 2.4.27 [14]. Due to different validation data from various laboratories with respect to the limit of quantification (LOQ), the highest value obtained with the respective method was taken as a harmonised LOQ, thereby establishing a unique basis for an appropriate evaluation. Table 2 shows the LOQs agreed by the working group.

Table 2 – *Limits of quantification (LOQ) for different elements*

Element	LOQ (ppm)
Arsenic	0.7
Cadmium	0.07
Cobalt	0.1
Mercury	0.02
Nickel	0.2
Lead	0.5
Vanadium	0.08 (herbal drugs)
	0.4 (essential oils)

Fresh plant material was dried prior to analysis. Certain variability might arise due to the different analytical methods used which should be taken into consideration in the assessment of heavy metals and the proposed limits.

Usually, knowledge of the number of samples *n* and the existence of a normal Gaussian distribution are required for statistical evaluation. However, as heavy metal content in herbal drugs does not normally show such a distribution, calculation of percentiles has been used [20].

In addition to the minimum and maximum values, the 90th percentile has also been used for assessing data. However, the 90th percentile refers to the number of positive samples (> LOQ), and for this reason, the overall frequency of findings is also relevant for the assessment. Consequently, the total number of samples and the percentage of positive samples for each element are given in Table 3.

Table 3 – *Percentage of positive samples for each element*

Element	Number of samples analysed	Number of positive samples	Percentage of positive samples
Arsenic	2 028	452	22.3 %
Cadmium	17 071	8 269	48.4 %
Cobalt	4 022	2 632	65.4 %
Lead	16 954	7 024	41.4 %
Mercury	13 277	2 640	19.9 %
Nickel	4 336	4 229	97.5 %
Vanadium	4 051	3 190	78.7 %

For evaluation of the 90th percentile of each heavy metal and each herbal drug, the obtained positive values were sorted by size. For calculation of the 90th percentiles, a number of positive samples of at least $n = 10$ is required, for example in the case of 10 values, the 90th percentile corresponds to the value of the 9th sample. The 90th percentile can be interpreted as the value for which any sample of the population shows a smaller value with a probability of 90 per cent. A more precise correlation with regard to the population can be achieved with an increasing number of samples. No evaluation of the 90th percentile was performed in cases with fewer than 10 positive samples.

5. RESULTS AND DISCUSSION

Appendix 1 shows the results for cadmium, lead and mercury, the number of samples analysed, the maximum value, the 90th percentile, the Ph. Eur. limit and the number and percentage of samples exceeding the Ph. Eur. limit for herbal drugs from conventional and organic production respectively. The following evaluation is performed for data sets (herbal drugs/essential oils and elemental impurities) for which at least 10 samples were available.

The Ph. Eur. limit was exceeded in 252 out of 15 778 samples analysed for cadmium (1.6 %), in 211 out of 15 630 samples analysed for lead (1.3 %) and in 37 out of 11 929 samples analysed for mercury (0.3 %).

This corresponds to 43 out of 326 herbal products for cadmium (13.2 %), 71 out of 324 for lead (21.9 %) and 11 out of 274 for mercury (4.0 %).

A comparison of the results of samples from conventional and organic production is shown in Table 4. In this comparison, only those herbal drugs for which samples of both conventional and organic production were available are presented. For both cadmium and lead, results of 174 products are available, while for mercury, results of 168 products are given.

The comparison shows substantially lower amounts for cadmium and lead in the organic samples. In the case of cadmium, 57.1 % of the conventional samples are above the LOQ compared to only 35.5 % of the organic samples, while 2.0 % of the conventional samples exceed the Ph. Eur. limit compared to only 0.6 % of the organic samples. For lead, 46.1 % of the conventional samples show a result above the LOQ against only 33.3 % of the organic samples, while 1.6 % of the conventional samples exceed the Ph. Eur. limit compared to only 0.6 % of the organic samples.

Table 4 – *Results for cadmium, lead and mercury from conventional and organic production*

			Conventional	Organic
Cadmium	Number of samples		8 370	5 086
	Number of samples	> LOQ	4 780	1 771
	Percentage of samples	> LOQ	57.1 %	35.5 %
	Number of samples	> Ph. Eur. limit	169	28
	Percentage of samples	> Ph. Eur. limit	2.0 %	0.6 %
Lead	Number of samples		8 249	5 086
	Number of samples	> LOQ	3 799	1 694
	Percentage of samples	> LOQ	46.1 %	33.3 %
	Number of samples	> Ph. Eur. limit	129	31
	Percentage of samples	> Ph. Eur. limit	1.6 %	0.6 %
Mercury	Number of samples		7 020	3 132
	Number of samples	> LOQ	1 305	612
	Percentage of samples	> LOQ	18.6 %	19.5 %
	Number of samples	> Ph. Eur. limit	21	10
	Percentage of samples	> Ph. Eur. limit	0.3 %	0.3 %

For mercury, however, there is no notable difference, with the proportion of positive samples being almost identical (18.6 % for conventional, 19.5 % for organic production), with the percentage of samples above the Ph. Eur. limit being the same for both collectives (0.3 %).

Possible reasons for the significantly lower values for cadmium and lead samples from organic production could be the selection of specific environmental conditions, e.g. the quality of soils in organic cultivation or the origin of the products, i.e. from areas with less heavy industry.

Appendix 2 shows the results for arsenic, cobalt, nickel and vanadium, the number of samples analysed, the maximum value, the 90th percentile, the maximum limit of ICH Q3D as well as the number and percentage of samples exceeding the Q3D limit for herbal drugs from conventional and organic production respectively. The following evaluation is performed for data sets (product and element) for which at least 10 samples were available. The limits derived from Option 1 of ICH Q3D are based on a maximum daily oral intake of 10 g of the product.

The Q3D limit was exceeded in 120 out of 1 249 samples analysed for arsenic (9.6 %), in 2 out of 3 245 samples for cobalt (0.06 %), in 31 out of 3 513 samples for nickel (0.9 %) and in 3 out of 3 260 samples for vanadium (0.09 %).

This corresponds to 17 out of 53 herbal products for arsenic (32.1 %), 2 out of 127 for cobalt (1.6 %), 12 out of 143 for nickel (8.4 %) and 1 product out of 128 for vanadium (0.8 %). In the case of arsenic, it is noted that 65 of the 120 cases concern brown algae (bladderwrack, chondria and seaweed), which contain organic-bound arsenic. The remaining 14 products showed findings above the limit in 55 out of 1 249 cases (4.4 %).

Hereafter, the results of the database evaluation will be discussed for each of the elements concerned.

5.1. Cadmium

For those herbal drugs where individual Ph. Eur. monographs set higher limits than the general monograph *Herbal drugs (1433)* (1.0 ppm, see Table 1), the following 90th percentiles have been calculated: *Willow bark (1583)* 2.74 ppm, *Tormentil (1478)* 2.15 ppm, *Fumitory (1869)* 1.36 ppm and bladderwrack, i.e. *Kelp (1426)*, 0.83 ppm. These results show that the exemptions laid down in the respective individual Ph. Eur. monographs are still justified. A further increase of the limit for willow bark and a decrease of the limit for kelp could be discussed.

For other herbal drugs where individual Ph. Eur. monographs exist, a 90th percentile above the general limit of 1.0 ppm has been calculated, e.g. 1.77 ppm for heart's ease (i.e. *Wild pansy (1855)*). As a Ph. Eur. monograph exists for this herbal drug, an individual exemption of

2.0 ppm for cadmium is considered useful. The following herbal drugs, for which no Ph. Eur. monograph exists, show 90th percentiles above 1.0 ppm: chicory root, cocoa beans, cocoa husks, elecampane rhizome, lily of the valley herb, Peruvian bark, spinach leaves, watercress herb and willow leaves.

5.2. Lead

For *Iceland moss (1439)* and stinging nettle root (i.e. *Nettle root (2538)*), where individual Ph. Eur. monographs set higher limits than the general monograph (5.0 ppm, see Table 1), the 90th percentiles were calculated as 7.28 ppm and 5.58 ppm respectively. These results demonstrate that the exemptions are still justified.

For some herbal drugs where a Ph. Eur. monograph exists, the 90th percentile exceeds the Ph. Eur. general limit of 5.0 ppm, e.g. 5.21 ppm for *Lime flower (0957)* (7.33 ppm in the case of organic production) or 12.6 ppm for *Belladonna leaf (0221)*. However, in both cases, an exemption does not need to be discussed as the percentile is not representative (16 out of 285 samples and 8 out of 30 samples exceeded the limit). The following herbal drugs, for which no Ph. Eur. monograph exists, show 90th percentiles above 5.0 ppm: arnica root, common wood sorrel plant, stinging nettle herb, sundew herb and watercress herb.

With regard to the limit for lead set in food supplements (3.0 ppm), 27 products show 90th percentiles of more than 3.0 ppm and less than 5.0 ppm. Although these products comply with the limits set for herbal drugs used for the production of medicinal products, there may be a problem when used as food supplements.

5.3. Mercury

For the following herbal drugs where individual Ph. Eur. monographs exist, the 90th percentiles exceed the Ph. Eur. limit of 0.1 ppm: *Ginkgo leaf (1828)* 0.21 ppm, *Ginger (1522)* 0.14 ppm (conventional production) and 0.16 ppm (organic production), with maximum values of 0.57 and 0.22 ppm respectively. For ginkgo leaf, a higher individual limit than 0.1 ppm might be discussed, but normally the herbal drug is used for extraction. For ginger rhizome, only a few results above the maximum limit of the Ph. Eur. were found (conventional production 3.9 %, organic production 8.8 %). For damiana leaf (no Ph. Eur. monograph), a 90th percentile of 0.22 ppm was calculated, with a maximum value of 0.28 ppm. However, the percentile is not deemed representative as only 12 samples were analysed.

It should be noted that ICH Q3D permits a limit of 3 ppm for a daily oral dose of 10 g of the final product.

5.4. Arsenic

As shown in Appendix 2, a high arsenic content is mainly found in marine organisms such as bladderwrack, seaweed and chondria. However, ICH Q3D puts a focus on inorganic arsenic due to its toxicity. This is in line with the USP monograph Elemental impurities – Limits [21] which includes a limit for inorganic arsenic of 1.5 ppm and the Ph. Eur. which permits a higher limit (maximum 90 ppm) for brown algae containing organic-bound arsenic, e.g. *Kelp (1426)*.

With regard to herbal drugs of non-marine origin, a total of 55 out of 1 249 samples (4.4 %), equating to 14 out of 50 herbal products (28 %) exceed the Option 1 limit of ICH Q3D (1.5 ppm). The highest value was found for cowslip (6.6 ppm) and the highest 90th percentile was calculated for *Nettle leaf (1897)* (2.96 ppm). The ICH Q3D limit, however, refers to the daily dose of the final product and not to the drug for which the transition rate has also to be taken into account. It could be considered that the transition rate of arsenic into infusions can be expected to be well below 100 %, as 1 experiment revealed a transition rate of 16 % [22] and another 29 % for a black tea sample with an exceptionally high arsenic content [23].

As a large number of findings on the arsenic content (77.7 %) are below the LOQ and, with the exception of marine products, only a few herbal drugs exceed the ICH Q3D limit, a general limit in herbal drugs is not considered necessary. Within an individual risk assessment according to Option 2 which also takes into account the daily dose, higher limits than 1.5 ppm could be

considered appropriate, for example in the case of a daily dose of 5 g of the final product, a limit of 3.0 ppm for the herbal drug could be a basis for discussion.

5.5. Cobalt

Only 2 out of 3 245 samples (0.06 %) exceeded the ICH Q3D limit (5 ppm related to a daily dose of 10 g of the final product). The highest value of 9.06 ppm and the highest 90th percentile of 3.77 ppm both relate to creeping thyme herb. For 71 out of 82 products, the 90th percentiles are lower than 1.0 ppm. As the findings above the limit can be regarded as outliers, the probability of herbal drugs exceeding the ICH Q3D limit for cobalt appears to be very low.

5.6. Nickel

The ICH Q3D limit (20 ppm related to a daily dose of 10 g of the final product) was exceeded by 31 out of 3 513 samples (0.9 %) from 12 different herbal products. More than 50 % of these samples relate to cocoa husks (8 out of 15 samples from organic production) and creeping thyme herb (8 out of 14 samples from conventional production). Other than these 2 products, the probability of herbal drugs exceeding the ICH Q3D limit for nickel appears to be low.

5.7. Vanadium

The ICH Q3D limit (10 ppm related to a daily dose of 10 g of the final product) was exceeded by 3 out of 16 samples of chondria (18.6 %) from a total of 3 260 samples (0.09 %). Apart from marine algae, the probability of herbal drugs exceeding the ICH Q3D limit for vanadium appears to be very low.

6. COMPARISON WITH FORMER EVALUATIONS

This publication results from continuous monitoring of the occurrence of potential contamination with heavy metals over many years. For this reason, it may be of interest to compare these more recent results to those evaluated and published some years earlier [2]. For selected herbal drugs, Appendix 3 displays the findings from the period 2008-2015 in comparison to the findings from the period 2002-2007.

In the new evaluation, the total number of samples analysed for which 90th percentiles could be calculated was 6 729 for cadmium, compared to 3 504 in the previous evaluation, 7 724 for lead compared to 3 581 and 1 723 for mercury compared to 461. The mean value of the 90th percentiles was 0.60 ppm for cadmium compared to 0.59 ppm previously, 2.44 ppm for lead compared to 3.05 ppm and 0.05 ppm for mercury compared to 0.04 ppm.

From this data, it can be concluded that for cadmium and mercury, there is no change with regard to the mean value of the 90th percentile, whereas in the case of lead, a decrease of the mean value of the 90th percentile by 20 % was observed. Appendix 3 also shows findings above the current Ph. Eur. limit and demonstrates changes (decreases and increases) of the 90th percentiles between the former and current evaluations. It is shown that for cadmium (77 cases), decreases of more than 10 % occur in 24 cases and more than 20 % in 16 cases, alongside increases of more than 10 % in 29 cases and more than 20 % in 20 cases. For lead (73 cases), decreases of more than 10 % can be seen in 28 cases and more than 20 % in 24 cases, alongside increases of more than 10 % in 31 cases and more than 20 % in 27 cases. For mercury (14 cases), decreases of more than 10 % are observed in 6 cases and more than 20 % in 4 cases, alongside increases of more than 10 % in 4 cases and more than 20 % in 4 cases.

7. ESSENTIAL OILS

As shown in Appendix 4, the results of heavy metals testing in 27 different essential oils (26 from conventional and 1 from organic production) were evaluated. In most cases the oils were tested for cadmium, lead and mercury, and altogether 721 sets of data were evaluated,

independent of their number (less than 10 in some cases). Positive results (i.e. above the LOQ) were found in 52 cases (7.2 %).

With regard to cadmium, lead and mercury, the Ph. Eur. limit (general monograph *Herbal drugs (1433)*) was exceeded in 2 cases (representing 0.3 % of the 687 samples analysed for these metals): 1 sample of rose oil showed a result of 8.65 ppm for lead (limit 5.0 ppm) and 1 sample of thyme oil showed a result of 0.13 ppm (limit 0.1 ppm) for mercury. As rose oil is used for pharmaceutical purposes only in rare cases and the result for thyme oil is still within the tolerance, it can be concluded that the probability of heavy metal contamination for distilled oils as well as for cold-pressed oils is low. For this reason, a general test for heavy metals does not seem to be required. Where applicable, depending on the production process, a test for heavy metals may be appropriate and introduced in an individual monograph.

For cobalt, nickel and vanadium, only a total of 34 samples from 6 different essential oils were examined. The ICH Q3D limit was not exceeded in any of these cases. No data is available for arsenic in essential oils.

The ICH Q3D limit for lead (calculated for herbal drugs) was exceeded in 5 samples from 3 products (myrrh oil, Pinus pinaster oil and rose oil). However, as herbal products are excluded from the scope of ICH Q3D, the Ph. Eur. limits described above apply for these essential oils. Furthermore, a daily dose of 10 g as referred to in ICH Q3D is not realistic for essential oils.

These findings on the heavy metal content in essential oils from the BAH database are also in line with other literature data. Distilled as well as cold-pressed essential oils are essentially free of heavy metals, and sporadic findings were predominantly close to the detection limit of the method used, but always well below the Ph. Eur. limit for herbal drugs [24-32].

Several studies examined the heavy metal content in essential oils produced from medicinal and aromatic plants grown on soils polluted with such elemental impurities. High concentrations of such contaminants in the soil did not result in transfer to the essential oils. Although it was shown that the medicinal plant accumulates some heavy metals from the soil, the distilled essential oils produced from these plants did not show elevated levels [24-30].

Studies on citrus essential oils revealed that essential oils obtained by cold pressing are also essentially devoid of toxic heavy metals and concentrations were usually lower than the limit of detection and always well below the Ph. Eur. limit on herbal drugs, as was also the case for the distilled oils [31, 32].

Together with the results given by the BAH database evaluation, it can be concluded that heavy metals present in the herbal drug are not transferred into the essential oil via distillation, and the risk of heavy metal contamination of cold-pressed essential oils also seems negligible. Although chapter 2.8.N12 of the German Pharmacopeia (DAB) [15] requires a limit test on heavy metals in essential oils, it can be concluded that there is no risk of heavy metal contamination in essential oils and a general test on heavy metals is not considered necessary, regardless of the production process.

8. OVERALL CONCLUSION

Evaluation of the data shows that the Ph. Eur. limits set for cadmium (1.0 ppm), lead (5.0 ppm) and mercury (0.1 ppm) in the general monographs and in individual monographs for several plants, e.g. those accumulating cadmium, are appropriate (with the exception of willow bark and kelp). Due to the rather low risk of contamination, skip lot testing for mercury may be justified. For lead and possibly for cadmium, skip lot testing may be possible based on an individual risk assessment.

Substantially lower amounts of cadmium and lead were observed in samples from organic production compared to those from conventional production. This is considered to be due to the origin of the herbal products and the related environmental conditions.

For elements without a general limit in the Ph. Eur. (arsenic, cobalt, nickel, vanadium), the following conclusions can be drawn: as a large number of findings on arsenic from the database

are below the LOQ, a general limit in herbal drugs is not required. However, as part of an individual risk assessment (whilst also taking into account the daily dose), limits higher than 1.5 ppm could be considered appropriate. The risk of herbal drugs exceeding the limits for cobalt, nickel or vanadium (the latter excluding marine algae) appears to be very low.

In general, the approach of ICH Q3D is considered helpful for the calculation of exposure in the final products, but not for assessment of herbal raw material. Irrespective of the fact that herbal products are excluded from the scope of ICH Q3D, the Ph. Eur. limits are regarded as sufficient to guarantee the safety of the material used for the production of herbal medicinal products.

A comparison of the findings from the period 2008–2015 in relation to those from 2002–2007 reveals a remarkably higher number of samples that were tested for cadmium, lead and mercury. For cadmium and mercury, the mean value of the 90th percentile remained almost unchanged, whereas for lead a slight decrease was observed. Altogether, 53 of 77 cases for cadmium, 59 of 73 cases for lead and 10 of 14 cases for mercury, showed increases or decreases of the 90th percentiles of at least 10 % for individual herbal drugs.

For essential oils, there is no apparent risk of heavy metal contamination as shown by evaluation of the database and other literature data. For this reason, a general test on heavy metals is not considered necessary.

9. PERSPECTIVES

The working group will continue collecting and evaluating data on heavy metals occurring in herbal drugs. As an update to previous contributions [1,2,33], further publications of the evaluation are planned on a regular basis in order to keep the overview relevant and up to date.

10. ACKNOWLEDGEMENTS

The authors wish to express their thanks to the following companies (in alphabetical order) which contributed to the current collection and evaluation of data on heavy metals:

Anklam Extrakt GmbH, Anklam, Germany
Biologische Heilmittel Heel GmbH, Baden-Baden, Germany
DHU Arzneimittel GmbH & Co. KG, Karlsruhe, Germany
Finzelberg GmbH & Co. KG, Andernach, Germany
Frutarom Switzerland Ltd, Wädenswil, Switzerland
Homöopathisches Laboratorium A. Pflüger GmbH & Co. KG, Rheda-Wiedenbrück, Germany
Martin Bauer GmbH & Co. KG, Vestenbergsgreuth, Germany
Mast-Jägermeister AG, Wolfenbüttel, Germany
Max Zeller Söhne AG, Romanshorn, Switzerland
SALUS Haus GmbH & Co. KG, Bruckmühl, Germany
WALA Heilmittel GmbH, Bad Boll/Eckwälden, Germany
Weleda AG, Schwäbisch Gmünd, Germany

Special thanks go to Mr Elmar Häfner, PhytoLab GmbH & Co. KG, for managing the database and for compiling the tables for this evaluation.

11. REFERENCES

1. Kabelitz L. Heavy metals in herbal drugs (Zur Schwermetallbelastung von Arznei- und Kräuterdrogen). *Pharm Ind* 1998; 60: 444-51.

2. Gasser U, Klier B, Kühn AV, Steinhoff B. Current Findings on the Heavy Metal Content in Herbal Drugs. *Pharmeur Sci Notes* 2009 (1): 37-50.

3. Herbal drugs, general monograph 1433. Ph. Eur. 9th edition. Strasbourg, France: Council of Europe; 2016 (vol 1).

4. Essential oils, general monograph 2098. Ph. Eur. 9th edition. Strasbourg, France: Council of Europe; 2016 (vol 1).

5. ICH guideline Q3D on elemental impurities. Step 5 version dated 25 July 2016. Ref: EMA/CHMP/ICH/353369/2013.

6. Kelp, monograph 1426. Ph. Eur. 9th edition. Strasbourg, France: Council of Europe; 2016 (vol 1).

7. Fumitory, monograph 1869. Ph. Eur. 9th edition. Strasbourg, France: Council of Europe; 2016 (vol 1).

8. Tormentil, monograph 1478. Ph. Eur. 9th edition. Strasbourg, France: Council of Europe; 2016 (vol 1).

9. Willow bark, monograph 1583. Ph. Eur. 9th edition. Strasbourg, France: Council of Europe; 2016 (vol 1).

10. Iceland moss, monograph 1439. Ph. Eur. 9th edition. Strasbourg, France: Council of Europe; 2016 (vol 1).

11. Nettle root, monograph 2538. Ph. Eur. 9th edition. Strasbourg, France: Council of Europe; 2016 (vol 1).

12. Herbal drugs for homoeopathic preparations, general monograph 2045. Ph. Eur. 9th edition. Strasbourg, France: Council of Europe; 2016 (vol 1).

13. Herbal drug extracts, general monograph 0765. Ph. Eur. 9th edition. Strasbourg, France: Council of Europe; 2016 (vol 1).

14. Heavy metals in herbal drugs and herbal drug preparations, general chapter 2.4.27. Ph. Eur. 9th edition. Strasbourg, France: Council of Europe; 2016 (vol 1).

15. Schwermetalle in ätherischen Ölen, chapter 2.8. N12. Deutsches Arzneibuch 2015. Stuttgart, Eschborn: Deutscher Apotheker Verlag, Govi-Verlag – Pharmazeutischer Verlag 2015.

16. Commission Regulation 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Union* L 364; 2006 Dec 20: 5-24.

17. Commission Regulation 396/2005 of 23 February 2005 on maximum residue levels in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC. *Official Journal of the European Union* L 070; 2005 Mar 16: 1-16.

18. Commission Regulation (EU) 2018/73 of 16 January 2018 amending Annexes II and III to Regulation (EC) No 396/2005 of the European Parliament and of the Council as regards maximum residue levels for mercury compounds in or on certain products. *Official Journal of the European Union* L 013; 2018 Jan 18: 8-20.

19. World Health Organization, Dept. of Technical Cooperation for Essential Drugs and Traditional Medicine. WHO guidelines for assessing quality of herbal medicines with reference to contaminants and residues. Geneva, Switzerland: WHO; 2007.

20. Hintergrundwerte für anorganische und organische Stoffe in Böden. 3rd edition. Bund-Länder-Arbeitsgemeinschaft Bodenschutz, LABO; 2003 [working group booklet].

21. Elemental impurities – Limits, general chapter <232>. First Supplement to USP 40, NF 35. Rockville, USA: United States Pharmacopeial Convention, Inc; 2016: 8065-8069.

22. Schulzki G, Nüßlein B, Sievers H. Transition rates of selected metals determined in various types of teas (*Camellia sinensis* L. Kuntze) and herbal/fruit infusions. *Food Chem* 2017; 215: 22-30.

23. Yuan C, Gao E, He B, Jiang G. Arsenic species and leaching characters in tea (*Camellia sinensis*). *Food Chem Toxicol* 2007; 45: 2381-2389.

24. Scora RW, Chang AC. Essential oil quality and heavy metal concentrations of peppermint grown on a municipal sludge-amended soil. *J Environ Qual* 1997;26:975-979.

25. Zheljazkov DV, Jekov D. Heavy metal content in some essential oils and plant extracts. *Acta Hort* 1996; 426: 427-433.

26. Zheljazkov DV, Craker LE, Xing B. Effects of Cd, Pb, and Cu on growth and essential oil contents in dill, peppermint, and basil. *Environ Exp Bot* 2006; 58: 9-16.

27. Blagojevic N, Damjanovic-Vratnica B, Vukasinovic-Pesic V, Durovic D. Heavy metals content in leaves and extracts of wild-growing salvia officinalis from Montenegro. *Polish J Environ Stud* 2009;18:167-173.

28. Zheljazkov DV, Jeliazkova E A, Kovacheva N, Dzhurmanski A. Metal uptake by medicinal plant species grown in soils contaminated by a smelter. Environ Exp Bot 2006; 64: 207-216.

29. Zheljazkov DV, Nielsen NE. Effect of heavy metals on peppermint and cornmint. *Plant and Soil* 1996; 78: 59-66.

30. Zheljazkov DV, Craker LE, Xing B *et al.* Aromatic plant production on metal contaminated soils. *Sci Total Environ* 2008; 385: 51-62.

31. La Pera L, Lo Curto R, Di Bella G, Dugo G. Determination of some heavy metals and selenium in Sicilian and Calabrian citrus essential oils using derivative stripping chronopotentiometry. *J Agric Food Chem* 2005; 53: 5084-5088.

32. Cautela D, Castaldo D, Santelli F *et al.* Survey of polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzo-p-furans (PCDFs), polychlorinated biphenyls (PCBs), and mineral components in Italian citrus cold-pressed essential oils. *J Agric Food Chem* 2007; 55: 1627-1637.

33. Guedon D, Brum M, Seigneuret JM, Impuretés des drogues végétales, préparations à base de drogues végétales et médicaments à base de plantes IV. Métaux lourds (toxiques); *STP pharma pratiques* 2008; 18; No. 3: 231-258.

Appendix 1. *Results for cadmium, lead and mercury in herbal drugs*

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	Ph. Eur. 9.0 limit		
						Max limit (ML)	No. of samples > ML	% Results > ML
Agrimony, herb	conv.	Cadmium	13	0.4	n.c.	1	0	0.0%
Agrimony, herb	conv.	Lead	13	1	n.c.	5	0	0.0%
Agrimony, herb	conv.	Mercury	13	0.03	n.c.	0.1	0	0.0%
Alder Buckthorn, bark	conv.	Cadmium	75	0.13	0.09	1	0	0.0%
Alder Buckthorn, bark	conv.	Lead	76	3.86	2.06	5	0	0.0%
Alder Buckthorn, bark	conv.	Mercury	62	0.02	n.c.	0.1	0	0.0%
Alfalfa, herb	conv.	Cadmium	10	0.07	n.c.	1	0	0.0%
Alfalfa, herb	conv.	Lead	10	0.5	n.c.	5	0	0.0%
Alfalfa, herb	conv.	Mercury	10	< 0.02	n.c.	0.1	0	0.0%
Allspice, fruits	conv.	Cadmium	11	< 0.07	n.c.	1	0	0.0%
Allspice, fruits	conv.	Lead	11	0.5	n.c.	5	0	0.0%
Almond, nuts	conv.	Cadmium	14	< 0.07	n.c.	1	0	0.0%
Almond, nuts	conv.	Lead	14	0.5	n.c.	5	0	0.0%
Almond, nuts	conv.	Mercury	14	< 0.02	n.c.	0.1	0	0.0%
Angelica, root	conv.	Cadmium	112	0.9	0.73	1	0	0.0%
Angelica, root	conv.	Lead	97	3.58	2.15	5	0	0.0%
Angelica, root	conv.	Mercury	83	0.09	n.c.	0.1	0	0.0%
Angelica, root	orga.	Cadmium	12	0.52	0.51	1	0	0.0%
Angelica, root	orga.	Lead	12	3.33	3.04	5	0	0.0%
Aniseed	conv.	Cadmium	37	0.12	n.c.	1	0	0.0%
Aniseed	conv.	Lead	37	2.12	n.c.	5	0	0.0%
Aniseed	conv.	Mercury	30	0.02	n.c.	0.1	0	0.0%
Aniseed	orga.	Cadmium	45	0.146	0.131	1	0	0.0%
Aniseed	orga.	Lead	46	4.39	n.c.	5	0	0.0%
Aniseed	orga.	Mercury	20	0.03	n.c.	0.1	0	0.0%
Apple, fruit	conv.	Cadmium	52	< 0.07	n.c.	1	0	0.0%
Apple, fruit	conv.	Lead	52	5.54	3.12	5	1	1.9%
Apple, fruit	conv.	Mercury	50	< 0.02	n.c.	0.1	0	0.0%
Apple, fruit	orga.	Cadmium	103	0.083	n.c.	1	0	0.0%
Apple, fruit	orga.	Lead	104	1.89	1.29	5	0	0.0%
Apple, fruit	orga.	Mercury	88	0.03	n.c.	0.1	0	0.0%
Arnica, flowers	conv.	Cadmium	120	1.25	0.49	1	1	0.8%
Arnica, flowers	conv.	Lead	117	23.6	2.76	5	3	2.6%
Arnica, flowers	conv.	Mercury	102	0.03	n.c.	0.1	0	0.0%
Arnica, root	conv.	Cadmium	18	1.06	0.52	1	1	5.6%
Arnica, root	conv.	Lead	18	26.3	10.9	5	5	27.8%
Arnica, root	conv.	Mercury	16	0.05	n.c.	0.1	0	0.0%
Barberry, root, bark	conv.	Cadmium	25	0.07	n.c.	1	0	0.0%
Barberry, root, bark	conv.	Lead	25	6.01	2.29	5	1	4.0%
Barberry, root, bark	conv.	Mercury	25	0.02	n.c.	0.1	0	0.0%
Bearberry, leaves	conv.	Cadmium	41	< 0.07	n.c.	1	0	0.0%
Bearberry, leaves	conv.	Lead	42	0.5	n.c.	5	0	0.0%
Bearberry, leaves	conv.	Mercury	40	0.03	n.c.	0.1	0	0.0%
Bearberry, leaves	orga.	Cadmium	20	< 0.07	n.c.	1	0	0.0%
Bearberry, leaves	orga.	Lead	20	0.608	n.c.	5	0	0.0%
Belladonna, leaves	conv.	Cadmium	26	0.88	0.73	1	0	0.0%
Belladonna, leaves	conv.	Lead	30	18.1	12.6	5	8	26.7%
Belladonna, leaves	conv.	Mercury	26	0.03	0.03	0.1	0	0.0%

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	Ph. Eur. 9.0 limit		
						Max limit (ML)	No. of samples > ML	% Results > ML
Bilberry, fruit	orga.	Cadmium	28	0.341	n.c.	1	0	0.0%
Bilberry, fruit	orga.	Lead	34	1.3	n.c.	5	0	0.0%
Bilberry, fruit	orga.	Mercury	11	< 0.02	n.c.	0.1	0	0.0%
Birch, bark	conv.	Cadmium	13	0.89	0.59	1	0	0.0%
Birch, bark	conv.	Lead	11	0.99	n.c.	5	0	0.0%
Birch, bark	conv.	Mercury	11	0.02	n.c.	0.1	0	0.0%
Birch, leaves	conv.	Cadmium	175	1.17	0.61	1	1	0.6%
Birch, leaves	conv.	Lead	177	34.2	3.02	5	4	2.3%
Birch, leaves	conv.	Mercury	167	0.07	0.03	0.1	0	0.0%
Birch, leaves	orga.	Cadmium	68	1.04	0.61	1	1	1.5%
Birch, leaves	orga.	Lead	68	7.03	0.993	5	1	1.5%
Birch, leaves	orga.	Mercury	24	0.02	n.c.	0.1	0	0.0%
Bitter Orange, peel	conv.	Cadmium	12	< 0.07	n.c.	1	0	0.0%
Bitter Orange, peel	conv.	Lead	13	0.5	n.c.	5	0	0.0%
Bittersweet, stems	conv.	Cadmium	16	0.31	0.24	1	0	0.0%
Bittersweet, stems	conv.	Lead	16	1.82	n.c.	5	0	0.0%
Bittersweet, stems	conv.	Mercury	16	0.03	n.c.	0.1	0	0.0%
Black Chokeberries	conv.	Cadmium	18	0.27	0.24	1	0	0.0%
Black Chokeberries	conv.	Lead	18	3.07	1.12	5	0	0.0%
Black Chokeberries	conv.	Mercury	17	0.02	n.c.	0.1	0	0.0%
Black cohosh, root	conv.	Cadmium	22	0.69	0.21	1	0	0.0%
Black cohosh, root	conv.	Lead	22	4.58	3.44	5	0	0.0%
Black cohosh, root	conv.	Mercury	22	< 0.02	n.c.	0.1	0	0.0%
Black Horehound, herb	conv.	Cadmium	24	0.25	n.c.	1	0	0.0%
Black Horehound, herb	conv.	Lead	24	5.77	n.c.	5	1	4.2%
Black Horehound, herb	conv.	Mercury	24	0.02	n.c.	0.1	0	0.0%
Blackberry, leaves	conv.	Cadmium	15	0.96	0.32	1	0	0.0%
Blackberry, leaves	conv.	Lead	15	4.09	n.c.	5	0	0.0%
Blackberry, leaves	conv.	Mercury	13	0.02	n.c.	0.1	0	0.0%
Blackberry, leaves	orga.	Cadmium	64	0.456	0.229	1	0	0.0%
Blackberry, leaves	orga.	Lead	64	5.64	1.36	5	1	1.6%
Blackberry, leaves	orga.	Mercury	33	0.03	n.c.	0.1	0	0.0%
Blackcurrant, fruit	conv.	Cadmium	25	0.25	n.c.	1	0	0.0%
Blackcurrant, fruit	conv.	Lead	25	1.46	0.94	5	0	0.0%
Blackcurrant, fruit	conv.	Mercury	24	< 0.02	n.c.	0.1	0	0.0%
Blackcurrant, fruit	orga.	Cadmium	25	< 0.07	n.c.	1	0	0.0%
Blackcurrant, fruit	orga.	Lead	26	1.1	n.c.	5	0	0.0%
Blackcurrant, fruit	orga.	Mercury	10	< 0.02	n.c.	0.1	0	0.0%
Blackcurrant, leaves	conv.	Cadmium	22	0.08	n.c.	1	0	0.0%
Blackcurrant, leaves	conv.	Lead	22	1.23	n.c.	5	0	0.0%
Blackcurrant, leaves	conv.	Mercury	22	0.04	n.c.	0.1	0	0.0%
Blackcurrant, leaves	orga.	Cadmium	16	< 0.07	n.c.	1	0	0.0%
Blackcurrant, leaves	orga.	Lead	16	2.13	n.c.	5	0	0.0%
Blackcurrant, leaves	orga.	Mercury	13	0.03	n.c.	0.1	0	0.0%
Bladderwrack	conv.	Cadmium	33	1	0.83	5	0	0.0%
Bladderwrack	conv.	Lead	33	1.58	1.13	4	0	0.0%
Bladderwrack	conv.	Mercury	33	0.1	0.08	0.1	0	0.0%
Blond Psyllium, husk	conv.	Cadmium	252	0.15	n.c.	1	0	0.0%
Blond Psyllium, husk	conv.	Lead	252	15	1.91	5	1	0.4%

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	Ph. Eur. 9.0 limit		
						Max limit (ML)	No. of samples > ML	% Results > ML
Blond Psyllium, husk	conv.	Mercury	252	< 0.02	n.c.	0.1	0	0.0%
Blond Psyllium, seeds	conv.	Cadmium	28	0.12	0.1	1	0	0.0%
Blond Psyllium, seeds	conv.	Lead	28	0.65	n.c.	5	0	0.0%
Blond Psyllium, seeds	conv.	Mercury	13	< 0.02	n.c.	0.1	0	0.0%
Blond Psyllium, seeds	orga.	Cadmium	22	0.131	n.c.	1	0	0.0%
Blond Psyllium, seeds	orga.	Lead	23	0.532	n.c.	5	0	0.0%
Blue Flag Iris, rhizome	conv.	Cadmium	42	0.29	0.18	1	0	0.0%
Blue Flag Iris, rhizome	conv.	Lead	42	0.5	n.c.	5	0	0.0%
Blue Flag Iris, rhizome	conv.	Mercury	42	0.02	n.c.	0.1	0	0.0%
Burdock, root	conv.	Cadmium	26	0.38	0.31	1	0	0.0%
Burdock, root	conv.	Lead	26	2.58	n.c.	5	0	0.0%
Burdock, root	conv.	Mercury	26	< 0.02	n.c.	0.1	0	0.0%
Burdock, root	orga.	Cadmium	17	14.3	0.41	1	1	5.9%
Burdock, root	orga.	Lead	16	3.31	2.69	5	0	0.0%
Burdock, root	orga.	Mercury	16	0.02	n.c.	0.1	0	0.0%
Butcher's Broom, rhizome	conv.	Cadmium	32	0.86	0.46	1	0	0.0%
Butcher's Broom, rhizome	conv.	Lead	37	8.5	3.67	5	2	5.4%
Butcher's Broom, rhizome	conv.	Mercury	27	0.04	0.03	0.1	0	0.0%
Butter Dock, leaves	conv.	Cadmium	91	0.5	0.3	1	0	0.0%
Butter Dock, leaves	conv.	Lead	25	4.7	2.8	5	0	0.0%
Butter Dock, leaves	conv.	Mercury	21	0.1	n.c.	0.1	0	0.0%
Butter Dock, root	conv.	Cadmium	29	1	0.7	1	0	0.0%
Butter Dock, root	conv.	Lead	18	8	4.6	5	2	11.1%
Butter Dock, root	conv.	Mercury	10	< 0.02	n.c.	0.1	0	0.0%
Californian Poppy, herb	conv.	Cadmium	13	1.18	0.55	1	1	7.7%
Californian Poppy, herb	conv.	Lead	13	2	n.c.	5	0	0.0%
Californian Poppy, herb	conv.	Mercury	13	0.03	n.c.	0.1	0	0.0%
Camomile, flowers	conv.	Cadmium	283	2.87	0.61	1	5	1.8%
Camomile, flowers	conv.	Lead	270	8.6	1.54	5	1	0.4%
Camomile, flowers	conv.	Mercury	240	0.05	0.03	0.1	0	0.0%
Camomile, flowers	orga.	Cadmium	178	1.21	0.577	1	4	2.2%
Camomile, flowers	orga.	Lead	179	9.22	1.27	5	2	1.1%
Camomile, flowers	orga.	Mercury	124	0.04	n.c.	0.1	0	0.0%
Camomile, herb	conv.	Cadmium	10	0.97	n.c.	1	0	0.0%
Camomile, herb	conv.	Lead	16	1.21	n.c.	5	0	0.0%
Camomile, herb	conv.	Mercury	10	< 0.02	n.c.	0.1	0	0.0%
Camomile, herb	orga.	Cadmium	17	0.49	0.39	1	0	0.0%
Camomile, herb	orga.	Lead	16	1.94	n.c.	5	0	0.0%
Camomile, herb	orga.	Mercury	16	0.02	n.c.	0.1	0	0.0%
Camomile, root	conv.	Cadmium	22	0.74	0.65	1	0	0.0%
Camomile, root	conv.	Lead	20	2.35	n.c.	5	0	0.0%
Camomile, root	conv.	Mercury	20	0.11	n.c.	0.1	1	5.0%
Camomile, seeds	orga.	Cadmium	17	0.49	n.c.	1	0	0.0%
Camomile, seeds	orga.	Lead	17	0.61	n.c.	5	0	0.0%
Camomile, seeds	orga.	Mercury	17	0.02	n.c.	0.1	0	0.0%
Caraway, seeds	conv.	Cadmium	33	0.19	n.c.	1	0	0.0%
Caraway, seeds	conv.	Lead	33	1.4	n.c.	5	0	0.0%
Caraway, seeds	conv.	Mercury	32	< 0.02	n.c.	0.1	0	0.0%
Caraway, seeds	orga.	Cadmium	45	0.28	0.24	1	0	0.0%

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	Ph. Eur. 9.0 limit		
						Max limit (ML)	No. of samples > ML	% Results > ML
Caraway, seeds	orga.	Lead	47	0.54	n.c.	5	0	0.0%
Caraway, seeds	orga.	Mercury	26	< 0.02	n.c.	0.1	0	0.0%
Cardamom, fruit	conv.	Cadmium	15	0.34	0.32	1	0	0.0%
Cardamom, fruit	conv.	Lead	15	0.5	n.c.	5	0	0.0%
Cardamom, fruit	conv.	Mercury	13	< 0.02	n.c.	0.1	0	0.0%
Cardamom, fruit	orga.	Cadmium	15	0.348	0.281	1	0	0.0%
Cardamom, fruit	orga.	Lead	15	0.931	n.c.	5	0	0.0%
Carrot	conv.	Cadmium	18	0.471	n.c.	1	0	0.0%
Carrot	conv.	Lead	18	0.5	n.c.	5	0	0.0%
Carrot	orga.	Cadmium	25	0.481	0.378	1	0	0.0%
Carrot	orga.	Lead	26	0.5	n.c.	5	0	0.0%
Carrot	orga.	Mercury	14	0.02	n.c.	0.1	0	0.0%
Cascara Sagrada, bark	conv.	Cadmium	14	0.08	n.c.	1	0	0.0%
Cascara Sagrada, bark	conv.	Lead	14	1.25	n.c.	5	0	0.0%
Cascara Sagrada, bark	conv.	Mercury	14	< 0.02	n.c.	0.1	0	0.0%
Cayenne Pepper, fruit	conv.	Cadmium	21	0.11	n.c.	1	0	0.0%
Cayenne Pepper, fruit	conv.	Lead	21	0.5	n.c.	5	0	0.0%
Cayenne Pepper, fruit	conv.	Mercury	21	< 0.02	n.c.	0.1	0	0.0%
Centaury, herb	conv.	Cadmium	26	0.18	n.c.	1	0	0.0%
Centaury, herb	conv.	Lead	27	1.14	1.12	5	0	0.0%
Centaury, herb	conv.	Mercury	15	< 0.02	n.c.	0.1	0	0.0%
Chaste tree, fruit	conv.	Cadmium	56	< 0.07	n.c.	1	0	0.0%
Chaste tree, fruit	conv.	Lead	59	1.42	1.18	5	0	0.0%
Chaste tree, fruit	conv.	Mercury	58	0.1	n.c.	0.1	0	0.0%
Chicory, herb	conv.	Cadmium	19	0.84	0.5	1	0	0.0%
Chicory, herb	conv.	Lead	19	1.86	n.c.	5	0	0.0%
Chicory, herb	conv.	Mercury	19	< 0.02	n.c.	0.1	0	0.0%
Chicory, root	conv.	Cadmium	18	1.22	1.12	1	2	11.1%
Chicory, root	conv.	Lead	17	1.36	n.c.	5	0	0.0%
Chicory, root	conv.	Mercury	10	< 0.02	n.c.	0.1	0	0.0%
Chicory, root	orga.	Cadmium	11	0.23	0.19	1	0	0.0%
Chicory, root	orga.	Lead	11	1.73	n.c.	5	0	0.0%
Chicory, root	orga.	Mercury	10	< 0.02	n.c.	0.1	0	0.0%
Chondria	conv.	Cadmium	16	0.6	0.363	1	0	0.0%
Chondria	conv.	Lead	16	3.35	n.c.	5	0	0.0%
Cinnamon, bark	conv.	Cadmium	55	0.59	0.53	1	0	0.0%
Cinnamon, bark	conv.	Lead	61	8.13	3.6	5	1	1.6%
Cinnamon, bark	conv.	Mercury	45	0.04	0.02	0.1	0	0.0%
Cinnamon, bark	orga.	Cadmium	68	0.362	0.33	1	0	0.0%
Cinnamon, bark	orga.	Lead	74	1.35	1.18	5	0	0.0%
Cinnamon, bark	orga.	Mercury	49	0.02	n.c.	0.1	0	0.0%
Cinquefoil, herb	conv.	Cadmium	22	0.41	0.24	1	0	0.0%
Cinquefoil, herb	conv.	Lead	22	1.48	0.91	5	0	0.0%
Cinquefoil, herb	conv.	Mercury	19	< 0.02	n.c.	0.1	0	0.0%
Clove, buds	conv.	Cadmium	40	0.3	n.c.	1	0	0.0%
Clove, buds	conv.	Lead	40	0.74	n.c.	5	0	0.0%
Clove, buds	conv.	Mercury	40	< 0.02	n.c.	0.1	0	0.0%
Clove, buds	orga.	Cadmium	18	< 0.07	n.c.	1	0	0.0%
Clove, buds	orga.	Lead	18	0.5	n.c.	5	0	0.0%

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	Ph. Eur. 9.0 limit		
						Max limit (ML)	No. of samples > ML	% Results > ML
Clove, buds	orga.	Mercury	13	< 0.02	n.c.	0.1	0	0.0%
Cocoa, beans	orga.	Cadmium	10	1.35	1.34	1	5	50.0%
Cocoa, beans	orga.	Lead	10	0.5	n.c.	5	0	0.0%
Cocoa, husks	orga.	Cadmium	52	1.95	1.43	1	7	13.5%
Cocoa, husks	orga.	Lead	48	12.8	1.33	5	1	2.1%
Cocoa, husks	orga.	Mercury	39	0.03	0.02	0.1	0	0.0%
Cola Nut, seeds	conv.	Cadmium	24	< 0.07	n.c.	1	0	0.0%
Cola Nut, seeds	conv.	Lead	22	0.521	n.c.	5	0	0.0%
Cola Nut, seeds	conv.	Mercury	12	< 0.02	n.c.	0.1	0	0.0%
Coltsfoot, leaves	conv.	Cadmium	23	0.74	0.35	1	0	0.0%
Coltsfoot, leaves	conv.	Lead	23	2.34	1.78	5	0	0.0%
Coltsfoot, leaves	conv.	Mercury	23	0.03	0.02	0.1	0	0.0%
Comfrey, root	conv.	Cadmium	11	0.16	n.c.	1	0	0.0%
Comfrey, root	conv.	Lead	11	2.21	n.c.	5	0	0.0%
Comfrey, root	conv.	Mercury	11	< 0.02	n.c.	0.1	0	0.0%
Common Beet	orga.	Cadmium	14	0.185	0.17	1	0	0.0%
Common Beet	orga.	Lead	14	0.5	n.c.	5	0	0.0%
Common Carline Thistl, root	conv.	Cadmium	11	0.877	0.711	1	0	0.0%
Common Carline Thistl, root	conv.	Lead	11	1.63	1.48	5	0	0.0%
Common Ivy, leaves and herb	conv.	Cadmium	81	0.81	0.51	1	0	0.0%
Common Ivy, leaves and herb	conv.	Lead	81	5.64	1.36	5	1	1.2%
Common Ivy, leaves and herb	conv.	Mercury	79	0.09	0.04	0.1	0	0.0%
Common Speedwell, herb	conv.	Cadmium	10	1.1	0.85	1	1	10.0%
Common Speedwell, herb	conv.	Lead	10	4.25	n.c.	5	0	0.0%
Common Speedwell, herb	conv.	Mercury	10	0.02	n.c.	0.1	0	0.0%
Common wood sorrel, Plant	conv.	Cadmium	16	0.94	0.38	1	0	0.0%
Common wood sorrel, Plant	conv.	Lead	15	6.83	6.52	5	3	20.0%
Common wood sorrel, Plant	conv.	Mercury	15	0.05	n.c.	0.1	0	0.0%
Common Wormwood, herb	conv.	Cadmium	60	0.836	0.63	1	0	0.0%
Common Wormwood, herb	conv.	Lead	53	3.28	1.31	5	0	0.0%
Common Wormwood, herb	conv.	Mercury	48	0.03	n.c.	0.1	0	0.0%
Common Wormwood, herb	orga.	Cadmium	25	0.558	0.362	1	0	0.0%
Common Wormwood, herb	orga.	Lead	25	0.64	n.c.	5	0	0.0%
Coriander, seeds	conv.	Cadmium	16	0.62	n.c.	1	0	0.0%
Coriander, seeds	conv.	Lead	16	0.5	n.c.	5	0	0.0%
Coriander, seeds	conv.	Mercury	15	< 0.02	n.c.	0.1	0	0.0%
Coriander, seeds	orga.	Cadmium	25	0.3	n.c.	1	0	0.0%
Coriander, seeds	orga.	Lead	25	0.58	n.c.	5	0	0.0%
Coriander, seeds	orga.	Mercury	20	< 0.02	n.c.	0.1	0	0.0%
Cornflower, flowers	orga.	Cadmium	25	0.408	0.199	1	0	0.0%

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	Ph. Eur. 9.0 limit		
						Max limit (ML)	No. of samples > ML	% Results > ML
Cornflower, flowers	orga.	Lead	25	0.92	n.c.	5	0	0.0%
Couch-Grass, rhizome	conv.	Cadmium	37	0.48	0.25	1	0	0.0%
Couch-Grass, rhizome	conv.	Lead	36	2.51	1.75	5	0	0.0%
Couch-Grass, rhizome	conv.	Mercury	24	< 0.02	n.c.	0.1	0	0.0%
Cowslip, Oxslip, flowers	conv.	Cadmium	14	< 0.07	n.c.	1	0	0.0%
Cowslip, Oxslip, flowers	conv.	Lead	14	3.82	n.c.	5	0	0.0%
Cowslip, Oxslip, flowers	conv.	Mercury	10	< 0.02	n.c.	0.1	0	0.0%
Cowslip, Oxslip, flowers	orga.	Cadmium	28	0.101	n.c.	1	0	0.0%
Cowslip, Oxslip, flowers	orga.	Lead	29	1.17	n.c.	5	0	0.0%
Cowslip, Oxslip, flowers	orga.	Mercury	13	< 0.02	n.c.	0.1	0	0.0%
Cowslip, Oxslip, root	conv.	Cadmium	85	0.63	0.25	1	0	0.0%
Cowslip, Oxslip, root	conv.	Lead	91	7.52	5	5	9	9.9%
Cowslip, Oxslip, root	conv.	Mercury	77	0.09	0.03	0.1	0	0.0%
Creeping Thyme, herb	conv.	Cadmium	33	0.55	0.311	1	0	0.0%
Creeping Thyme, herb	conv.	Lead	33	3.66	1.86	5	0	0.0%
Creeping Thyme, herb	conv.	Mercury	24	0.02	n.c.	0.1	0	0.0%
Curcuma, root	conv.	Cadmium	35	0.66	0.277	1	0	0.0%
Curcuma, root	conv.	Lead	35	0.735	n.c.	5	0	0.0%
Curcuma, root	conv.	Mercury	21	0.02	n.c.	0.1	0	0.0%
Curled Mint, leaves	orga.	Cadmium	12	< 0.07	n.c.	1	0	0.0%
Curled Mint, leaves	orga.	Lead	12	0.72	n.c.	5	0	0.0%
Curled Mint, leaves	orga.	Mercury	12	0.03	n.c.	0.1	0	0.0%
Daisy, flowers	orga.	Cadmium	15	0.728	0.417	1	0	0.0%
Daisy, flowers	orga.	Lead	15	17.2	n.c.	5	1	6.7%
Damiana, leaves	conv.	Cadmium	14	0.27	0.25	1	0	0.0%
Damiana, leaves	conv.	Lead	12	0.61	n.c.	5	0	0.0%
Damiana, leaves	conv.	Mercury	12	0.28	0.22	0.1	4	33.3%
Dandelion, herb	orga.	Cadmium	80	0.685	0.433	1	0	0.0%
Dandelion, herb	orga.	Lead	80	5.85	2.71	5	1	1.3%
Dandelion, herb	orga.	Mercury	34	0.03	n.c.	0.1	0	0.0%
Dandelion, herb, root	conv.	Cadmium	89	0.95	0.52	1	0	0.0%
Dandelion, herb, root	conv.	Lead	86	14	3.43	5	3	3.5%
Dandelion, herb, root	conv.	Mercury	75	0.04	0.02	0.1	0	0.0%
Dandelion, herb, root	orga.	Cadmium	26	0.493	0.409	1	0	0.0%
Dandelion, herb, root	orga.	Lead	26	1.55	1.41	5	0	0.0%
Dandelion, root	conv.	Cadmium	42	0.85	0.42	1	0	0.0%
Dandelion, root	conv.	Lead	40	3.76	1.57	5	0	0.0%
Dandelion, root	conv.	Mercury	39	0.02	n.c.	0.1	0	0.0%
Dandelion, root	orga.	Cadmium	63	0.567	0.37	1	0	0.0%
Dandelion, root	orga.	Lead	62	4.17	1.93	5	0	0.0%
Dandelion, root	orga.	Mercury	45	0.02	n.c.	0.1	0	0.0%
Dead Nettle, flowers	conv.	Cadmium	18	< 0.07	n.c.	1	0	0.0%
Dead Nettle, flowers	conv.	Lead	21	3.61	1.5	5	0	0.0%
Devil's claw, root	conv.	Cadmium	154	0.126	0.12	1	0	0.0%
Devil's claw, root	conv.	Lead	155	1.28	0.85	5	0	0.0%
Devil's claw, root	conv.	Mercury	122	0.04	n.c.	0.1	0	0.0%
Dwarf montain pine, shoot	conv.	Cadmium	12	0.266	0.244	1	0	0.0%
Dwarf montain pine, shoot	conv.	Lead	12	1.02	n.c.	5	0	0.0%
Echinacea, herb	conv.	Cadmium	114	0.38	0.12	1	0	0.0%

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	Ph. Eur. 9.0 limit		
						Max limit (ML)	No. of samples > ML	% Results > ML
Echinacea, herb	conv.	Lead	121	3.42	1.18	5	0	0.0%
Echinacea, herb	conv.	Mercury	112	0.04	0.03	0.1	0	0.0%
Echinacea, herb	orga.	Cadmium	20	< 0.07	n.c.	1	0	0.0%
Echinacea, herb	orga.	Lead	22	0.506	n.c.	5	0	0.0%
Echinacea, herb	orga.	Mercury	17	0.02	n.c.	0.1	0	0.0%
Echinacea, root	conv.	Cadmium	47	0.94	0.47	1	0	0.0%
Echinacea, root	conv.	Lead	38	4.12	2.5	5	0	0.0%
Echinacea, root	conv.	Mercury	30	0.03	n.c.	0.1	0	0.0%
Elder, flowers	conv.	Cadmium	51	0.2	n.c.	1	0	0.0%
Elder, flowers	conv.	Lead	57	9.2	3.09	5	1	1.8%
Elder, flowers	conv.	Mercury	46	0.02	0.02	0.1	0	0.0%
Elder, flowers	orga.	Cadmium	125	< 0.07	n.c.	1	0	0.0%
Elder, flowers	orga.	Lead	128	16.3	2.38	5	5	3.9%
Elder, flowers	orga.	Mercury	94	0.02	n.c.	0.1	0	0.0%
Elder, fruit	conv.	Cadmium	12	< 0.07	n.c.	1	0	0.0%
Elder, fruit	conv.	Lead	12	2.14	n.c.	5	0	0.0%
Elder, fruit	conv.	Mercury	12	< 0.02	n.c.	0.1	0	0.0%
Elder, fruit	orga.	Cadmium	26	< 0.07	n.c.	1	0	0.0%
Elder, fruit	orga.	Lead	28	0.91	n.c.	5	0	0.0%
Elder, fruit	orga.	Mercury	10	< 0.02	n.c.	0.1	0	0.0%
Elecampane, rhizome	conv.	Cadmium	37	1.59	1.13	1	6	16.2%
Elecampane, rhizome	conv.	Lead	28	1.14	n.c.	5	0	0.0%
Elecampane, rhizome	conv.	Mercury	25	< 0.02	n.c.	0.1	0	0.0%
Ergot of Rye	conv.	Cadmium	14	0.18	n.c.	1	0	0.0%
Ergot of Rye	conv.	Lead	14	0.5	n.c.	5	0	0.0%
Ergot of Rye	conv.	Mercury	14	< 0.02	n.c.	0.1	0	0.0%
Eucalyptus, leaves	conv.	Cadmium	20	< 0.07	n.c.	1	0	0.0%
Eucalyptus, leaves	conv.	Lead	20	1.89	n.c.	5	0	0.0%
Eucalyptus, leaves	conv.	Mercury	20	0.1	0.05	0.1	0	0.0%
Eucalyptus, leaves	orga.	Cadmium	15	0.077	n.c.	1	0	0.0%
Eucalyptus, leaves	orga.	Lead	17	0.5	n.c.	5	0	0.0%
Eyebright, herb	conv.	Cadmium	66	1.58	0.965	1	4	6.1%
Eyebright, herb	conv.	Lead	58	3.39	1.5	5	0	0.0%
Eyebright, herb	conv.	Mercury	49	0.02	n.c.	0.1	0	0.0%
Fennel, seeds	conv.	Cadmium	39	0.16	n.c.	1	0	0.0%
Fennel, seeds	conv.	Lead	39	0.54	n.c.	5	0	0.0%
Fennel, seeds	conv.	Mercury	27	0.02	n.c.	0.1	0	0.0%
Fennel, seeds	orga.	Cadmium	171	0.31	0.25	1	0	0.0%
Fennel, seeds	orga.	Lead	174	0.601	n.c.	5	0	0.0%
Fennel, seeds	orga.	Mercury	103	0.02	n.c.	0.1	0	0.0%
Fenugreek, seeds	orga.	Cadmium	11	< 0.07	n.c.	1	0	0.0%
Fenugreek, seeds	orga.	Lead	11	0.5	n.c.	5	0	0.0%
Fenugreek, seeds	orga.	Mercury	11	< 0.02	n.c.	0.1	0	0.0%
Fig tree, fruit	conv.	Cadmium	20	0.073	n.c.	1	0	0.0%
Fig tree, fruit	conv.	Lead	25	0.5	n.c.	5	0	0.0%
Fig tree, fruit	conv.	Mercury	14	0.022	n.c.	0.1	0	0.0%
Fig tree, fruit	orga.	Cadmium	11	0.497	n.c.	1	0	0.0%
Fig tree, fruit	orga.	Lead	14	0.5	n.c.	5	0	0.0%
Florence Fennel, seeds	orga.	Cadmium	60	< 0.07	n.c.	1	0	0.0%

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	Ph. Eur. 9.0 limit		
						Max limit (ML)	No. of samples > ML	% Results > ML
Florence Fennel, seeds	orga.	Lead	60	0.51	n.c.	5	0	0.0%
Florence Fennel, seeds	orga.	Mercury	48	0.02	n.c.	0.1	0	0.0%
Frankincense	conv.	Cadmium	19	< 0.07	n.c.	1	0	0.0%
Frankincense	conv.	Lead	20	0.5	n.c.	5	0	0.0%
Frankincense	conv.	Mercury	20	0.02	n.c.	0.1	0	0.0%
French Bean, pods	conv.	Cadmium	10	0.15	n.c.	1	0	0.0%
French Bean, pods	conv.	Lead	10	0.5	n.c.	5	0	0.0%
Fumitory, herb	conv.	Cadmium	107	2.91	1.36	1.5	7	6.5%
Fumitory, herb	conv.	Lead	92	11.8	4.22	5	4	4.3%
Fumitory, herb	conv.	Mercury	88	0.03	n.c.	0.1	0	0.0%
Garlic, bulb	conv.	Cadmium	30	0.12	n.c.	1	0	0.0%
Garlic, bulb	conv.	Lead	30	15.2	n.c.	5	1	3.3%
Garlic, bulb	conv.	Mercury	25	0.04	n.c.	0.1	0	0.0%
Giant Goldenrod, herb	conv.	Cadmium	29	0.54	0.39	1	0	0.0%
Giant Goldenrod, herb	conv.	Lead	29	0.92	n.c.	5	0	0.0%
Giant Goldenrod, herb	conv.	Mercury	28	0.02	n.c.	0.1	0	0.0%
Giant Goldenrod, herb	orga.	Cadmium	11	0.711	n.c.	1	0	0.0%
Giant Goldenrod, herb	orga.	Lead	12	0.5	n.c.	5	0	0.0%
Ginger, rhizome	conv.	Cadmium	92	0.65	0.41	1	0	0.0%
Ginger, rhizome	conv.	Lead	75	2.6	1.16	5	0	0.0%
Ginger, rhizome	conv.	Mercury	51	0.57	0.14	0.1	2	3.9%
Ginger, rhizome	orga.	Cadmium	86	0.24	0.12	1	0	0.0%
Ginger, rhizome	orga.	Lead	87	1.67	0.915	5	0	0.0%
Ginger, rhizome	orga.	Mercury	80	0.22	0.16	0.1	7	8.8%
Ginkgo, leaves	conv.	Cadmium	61	0.38	0.12	1	0	0.0%
Ginkgo, leaves	conv.	Lead	64	8.11	4.64	5	5	7.8%
Ginkgo, leaves	conv.	Mercury	68	0.21	0.21	0.1	14	20.6%
Ginkgo, leaves	orga.	Cadmium	10	0.12	n.c.	1	0	0.0%
Ginkgo, leaves	orga.	Lead	10	4.8	3.56	5	0	0.0%
Ginseng, root	conv.	Cadmium	77	0.26	0.17	1	0	0.0%
Ginseng, root	conv.	Lead	75	1.7	n.c.	5	0	0.0%
Ginseng, root	conv.	Mercury	68	0.1	0.02	0.1	0	0.0%
Globe Artichoke, leaves	conv.	Cadmium	130	0.58	0.4	1	0	0.0%
Globe Artichoke, leaves	conv.	Lead	129	2.82	1.8	5	0	0.0%
Globe Artichoke, leaves	conv.	Mercury	76	0.12	0.02	0.1	1	1.3%
Globe Artichoke, leaves	orga.	Cadmium	11	0.306	0.278	1	0	0.0%
Globe Artichoke, leaves	orga.	Lead	12	1.48	n.c.	5	0	0.0%
Golden Rod, herb	conv.	Cadmium	105	1.03	0.85	1	2	1.9%
Golden Rod, herb	conv.	Lead	80	2.5	2.34	5	0	0.0%
Golden Rod, herb	conv.	Mercury	75	0.02	n.c.	0.1	0	0.0%
Golden Rod, herb	orga.	Cadmium	18	1.15	0.79	1	1	5.6%
Golden Rod, herb	orga.	Lead	17	0.538	n.c.	5	0	0.0%
Goosegrass, herb	orga.	Cadmium	13	0.38	0.38	1	0	0.0%
Goosegrass, herb	orga.	Lead	13	1.15	n.c.	5	0	0.0%
Goosegrass, herb	orga.	Mercury	13	0.02	n.c.	0.1	0	0.0%
Grapes, seeds	conv.	Cadmium	27	< 0.07	n.c.	1	0	0.0%
Grapes, seeds	conv.	Lead	27	0.8	n.c.	5	0	0.0%
Grapes, seeds	conv.	Mercury	27	0.02	n.c.	0.1	0	0.0%
Greater Celandine, herb	conv.	Cadmium	13	0.19	n.c.	1	0	0.0%

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	Ph. Eur. 9.0 limit		
						Max limit (ML)	No. of samples > ML	% Results > ML
Greater Celandine, herb	conv.	Lead	13	6.17	2.57	5	1	7.7%
Greater Celandine, herb	conv.	Mercury	13	0.02	n.c.	0.1	0	0.0%
Greater Celandine, root	conv.	Cadmium	16	0.22	0.13	1	0	0.0%
Greater Celandine, root	conv.	Lead	16	4.78	4.38	5	0	0.0%
Greater Celandine, root	conv.	Mercury	16	0.02	n.c.	0.1	0	0.0%
Guar, gum	conv.	Cadmium	15	< 0.07	n.c.	1	0	0.0%
Guar, gum	conv.	Lead	15	0.65	n.c.	5	0	0.0%
Guar, gum	conv.	Mercury	15	< 0.02	n.c.	0.1	0	0.0%
Guarana, seeds	conv.	Cadmium	11	< 0.07	n.c.	1	0	0.0%
Guarana, seeds	conv.	Lead	11	0.5	n.c.	5	0	0.0%
Guarana, seeds	conv.	Mercury	10	< 0.02	n.c.	0.1	0	0.0%
Hawthorn, flowers	conv.	Cadmium	23	0.2	0.18	1	0	0.0%
Hawthorn, flowers	conv.	Lead	22	2.42	1.9	5	0	0.0%
Hawthorn, flowers	conv.	Mercury	23	0.02	n.c.	0.1	0	0.0%
Hawthorn, fruit	conv.	Cadmium	65	0.1	n.c.	1	0	0.0%
Hawthorn, fruit	conv.	Lead	66	0.73	n.c.	5	0	0.0%
Hawthorn, fruit	conv.	Mercury	59	< 0.02	n.c.	0.1	0	0.0%
Hawthorn, fruit	orga.	Cadmium	19	0.07	n.c.	1	0	0.0%
Hawthorn, fruit	orga.	Lead	20	0.5	n.c.	5	0	0.0%
Hawthorn, fruit	orga.	Mercury	11	< 0.02	n.c.	0.1	0	0.0%
Hawthorn, leaves and flowers	conv.	Cadmium	143	0.505	0.18	1	0	0.0%
Hawthorn, leaves and flowers	conv.	Lead	177	34.3	3.54	5	11	6.2%
Hawthorn, leaves and flowers	conv.	Mercury	121	0.02	0.02	0.1	0	0.0%
Hawthorn, leaves and flowers	orga.	Cadmium	43	0.24	0.215	1	0	0.0%
Hawthorn, leaves and flowers	orga.	Lead	44	2.27	1.1	5	0	0.0%
Hawthorn, leaves and flowers	orga.	Mercury	16	< 0.02	n.c.	0.1	0	0.0%
Heart's Ease, herb	conv.	Cadmium	55	2.15	1.77	1	36	65.5%
Heart's Ease, herb	conv.	Lead	32	3.28	2.4	5	0	0.0%
Heart's Ease, herb	conv.	Mercury	24	< 0.02	n.c.	0.1	0	0.0%
Henna, leaves	conv.	Cadmium	10	0.09	n.c.	1	0	0.0%
Henna, leaves	conv.	Lead	10	2.24	n.c.	5	0	0.0%
Henna, leaves	conv.	Mercury	10	0.13	n.c.	0.1	1	10.0%
Hibiscus, flowers	conv.	Cadmium	178	0.37	0.15	1	0	0.0%
Hibiscus, flowers	conv.	Lead	121	3.92	1.63	5	0	0.0%
Hibiscus, flowers	conv.	Mercury	115	0.03	n.c.	0.1	0	0.0%
Hibiscus, flowers	orga.	Cadmium	87	0.25	0.132	1	0	0.0%
Hibiscus, flowers	orga.	Lead	88	0.97	n.c.	5	0	0.0%
Hibiscus, flowers	orga.	Mercury	71	0.02	n.c.	0.1	0	0.0%
Holy Thistle, herb	conv.	Cadmium	10	0.268	n.c.	1	0	0.0%
Holy Thistle, herb	conv.	Lead	11	0.5	n.c.	5	0	0.0%
Holy Thistle, herb	orga.	Cadmium	18	0.818	0.645	1	0	0.0%
Holy Thistle, herb	orga.	Lead	18	2.63	n.c.	5	0	0.0%
Holy Thistle, herb	orga.	Mercury	14	< 0.02	n.c.	0.1	0	0.0%
Honey	conv.	Cadmium	19	< 0.07	n.c.	1	0	0.0%
Honey	conv.	Lead	19	0.5	n.c.	5	0	0.0%

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	Ph. Eur. 9.0 limit		
						Max limit (ML)	No. of samples > ML	% Results > ML
Honey	conv.	Mercury	16	< 0.02	n.c.	0.1	0	0.0%
Honey Bush, herb	orga.	Cadmium	28	< 0.07	n.c.	1	0	0.0%
Honey Bush, herb	orga.	Lead	28	0.51	n.c.	5	0	0.0%
Honey Bush, herb	orga.	Mercury	20	0.06	n.c.	0.1	0	0.0%
Hops	conv.	Cadmium	194	0.41	n.c.	1	0	0.0%
Hops	conv.	Lead	193	3.97	1.63	5	0	0.0%
Hops	conv.	Mercury	181	0.1	0.07	0.1	0	0.0%
Hops	orga.	Cadmium	26	< 0.07	n.c.	1	0	0.0%
Hops	orga.	Lead	27	1.6	n.c.	5	0	0.0%
Hops	orga.	Mercury	16	0.02	n.c.	0.1	0	0.0%
Horehound, herb	conv.	Cadmium	44	0.18	0.15	1	0	0.0%
Horehound, herb	conv.	Lead	44	3.87	1.64	5	0	0.0%
Horehound, herb	conv.	Mercury	44	0.02	0.02	0.1	0	0.0%
Horse-Chestnut, bark	conv.	Cadmium	19	0.08	n.c.	1	0	0.0%
Horse-Chestnut, bark	conv.	Lead	19	4.09	3.57	5	0	0.0%
Horse-Chestnut, bark	conv.	Mercury	19	0.02	n.c.	0.1	0	0.0%
Horse-Chestnut, leaves	conv.	Cadmium	10	0.15	n.c.	1	0	0.0%
Horse-Chestnut, leaves	conv.	Lead	12	2.21	n.c.	5	0	0.0%
Horse-Chestnut, seeds	conv.	Cadmium	78	< 0.07	n.c.	1	0	0.0%
Horse-Chestnut, seeds	conv.	Lead	78	0.91	n.c.	5	0	0.0%
Horse-Chestnut, seeds	conv.	Mercury	77	< 0.02	n.c.	0.1	0	0.0%
Horsetail, herb	conv.	Cadmium	83	0.32	0.15	1	0	0.0%
Horsetail, herb	conv.	Lead	84	9.24	1.04	5	1	1.2%
Horsetail, herb	conv.	Mercury	82	0.04	n.c.	0.1	0	0.0%
Horsetail, herb	orga.	Cadmium	60	1.44	0.184	1	1	1.7%
Horsetail, herb	orga.	Lead	60	56.9	2.61	5	1	1.7%
Horsetail, herb	orga.	Mercury	18	< 0.02	n.c.	0.1	0	0.0%
Hyssop, herb	orga.	Cadmium	14	0.374	n.c.	1	0	0.0%
Hyssop, herb	orga.	Lead	15	0.98	n.c.	5	0	0.0%
Hyssop, herb	orga.	Mercury	12	0.029	n.c.	0.1	0	0.0%
Iceland moss, herb	conv.	Cadmium	105	1.57	0.49	1	2	1.9%
Iceland moss, herb	conv.	Lead	112	13.4	7.28	10	2	1.8%
Iceland moss, herb	conv.	Mercury	96	0.04	0.03	0.1	0	0.0%
Ignatius bean, seeds	conv.	Cadmium	11	< 0.07	n.c.	1	0	0.0%
Ignatius bean, seeds	conv.	Lead	11	0.5	n.c.	5	0	0.0%
Ignatius bean, seeds	conv.	Mercury	11	< 0.02	n.c.	0.1	0	0.0%
Indian berry, fruits	conv.	Mercury	11	0.57	n.c.	0.1	4	36.4%
Ipecacuanha, root	conv.	Cadmium	12	0.07	n.c.	1	0	0.0%
Ipecacuanha, root	conv.	Lead	12	1.09	n.c.	5	0	0.0%
Ipecacuanha, root	conv.	Mercury	12	0.04	n.c.	0.1	0	0.0%
Java Tea, leaves	conv.	Cadmium	53	1.09	0.09	1	1	1.9%
Java Tea, leaves	conv.	Lead	56	3.66	2.44	5	0	0.0%
Java Tea, leaves	conv.	Mercury	44	0.08	0.03	0.1	0	0.0%
Juniper, fruit	conv.	Cadmium	27	0.15	0.12	1	0	0.0%
Juniper, fruit	conv.	Lead	26	0.69	n.c.	5	0	0.0%
Juniper, fruit	conv.	Mercury	22	0.02	0.02	0.1	0	0.0%
Juniper, fruit	orga.	Cadmium	23	0.16	n.c.	1	0	0.0%
Juniper, fruit	orga.	Lead	25	0.5	n.c.	5	0	0.0%
Knotgrass, herb	conv.	Cadmium	20	0.228	0.133	1	0	0.0%

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	Ph. Eur. 9.0 limit		
						Max limit (ML)	No. of samples > ML	% Results > ML
Knotgrass, herb	conv.	Lead	20	8.39	4.29	5	1	5.0%
Ladies Mantle, herb	conv.	Cadmium	21	0.424	0.293	1	0	0.0%
Ladies Mantle, herb	conv.	Lead	22	3.01	0.84	5	0	0.0%
Ladies Mantle, herb	orga.	Cadmium	34	0.9	0.285	1	0	0.0%
Ladies Mantle, herb	orga.	Lead	35	3.73	n.c.	5	0	0.0%
Ladies Mantle, herb	orga.	Mercury	11	< 0.02	n.c.	0.1	0	0.0%
Lapacho, bark	conv.	Cadmium	34	< 0.07	n.c.	1	0	0.0%
Lapacho, bark	conv.	Lead	35	2.19	n.c.	5	0	0.0%
Lapacho, bark	conv.	Mercury	25	0.02	n.c.	0.1	0	0.0%
Lavender, flowers	conv.	Cadmium	19	0.07	n.c.	1	0	0.0%
Lavender, flowers	conv.	Lead	19	5.99	2.56	5	1	5.3%
Lavender, flowers	conv.	Mercury	17	0.02	n.c.	0.1	0	0.0%
Lavender, flowers	orga.	Cadmium	49	0.203	n.c.	1	0	0.0%
Lavender, flowers	orga.	Lead	53	6.31	1.86	5	1	1.9%
Lavender, flowers	orga.	Mercury	22	< 0.02	n.c.	0.1	0	0.0%
Lemon Balm, leaves	conv.	Cadmium	122	0.29	0.11	1	0	0.0%
Lemon Balm, leaves	conv.	Lead	125	2.51	1.4	5	0	0.0%
Lemon Balm, leaves	conv.	Mercury	113	0.05	0.02	0.1	0	0.0%
Lemon Balm, leaves	orga.	Cadmium	277	0.14	0.14	1	0	0.0%
Lemon Balm, leaves	orga.	Lead	282	5.33	2.55	5	1	0.4%
Lemon Balm, leaves	orga.	Mercury	234	0.1	0.03	0.1	0	0.0%
Lemon Verbena, herb	orga.	Cadmium	40	0.328	n.c.	1	0	0.0%
Lemon Verbena, herb	orga.	Lead	40	1.81	n.c.	5	0	0.0%
Lemon Verbena, herb	orga.	Mercury	23	0.04	0.034	0.1	0	0.0%
Lemon, peel (C. limon)	orga.	Cadmium	17	0.09	n.c.	1	0	0.0%
Lemon, peel (C. limon)	orga.	Lead	17	0.5	n.c.	5	0	0.0%
Lemon, peel (C. limon)	orga.	Mercury	14	< 0.02	n.c.	0.1	0	0.0%
Lemonade Tree, fruit	conv.	Cadmium	14	< 0.07	n.c.	1	0	0.0%
Lemonade Tree, fruit	conv.	Lead	14	4.22	n.c.	5	0	0.0%
Lemonade Tree, fruit	conv.	Mercury	12	0.08	n.c.	0.1	0	0.0%
Lemongrass, leaves	orga.	Cadmium	95	0.11	n.c.	1	0	0.0%
Lemongrass, leaves	orga.	Lead	95	0.78	n.c.	5	0	0.0%
Lemongrass, leaves	orga.	Mercury	75	0.03	n.c.	0.1	0	0.0%
Lily of the Valley, herb	conv.	Cadmium	20	1.44	1.23	1	4	20.0%
Lily of the Valley, herb	conv.	Lead	15	2.08	n.c.	5	0	0.0%
Lily of the Valley, herb	conv.	Mercury	15	0.03	n.c.	0.1	0	0.0%
Lime, flowers	conv.	Cadmium	167	0.47	0.17	1	0	0.0%
Lime, flowers	conv.	Lead	183	15.4	5.21	5	13	7.1%
Lime, flowers	conv.	Mercury	58	0.03	0.02	0.1	0	0.0%
Lime, flowers	orga.	Cadmium	100	0.2	0.16	1	0	0.0%
Lime, flowers	orga.	Lead	102	13.2	7.33	5	3	2.9%
Lime, flowers	orga.	Mercury	61	< 0.02	n.c.	0.1	0	0.0%
Lime, leaves	orga.	Cadmium	34	0.212	0.181	1	0	0.0%
Lime, leaves	orga.	Lead	36	5.66	n.c.	5	1	2.8%
Linseed	conv.	Cadmium	10	0.41	0.401	0.5	0	0.0%
Linseed	orga.	Cadmium	23	0.16	0.15	0.5	0	0.0%
Linseed	orga.	Lead	22	0.5	n.c.	5	0	0.0%
Linseed	orga.	Mercury	18	0.02	n.c.	0.1	0	0.0%
Liquorice, root	conv.	Cadmium	159	0.17	n.c.	1	0	0.0%

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	Ph. Eur. 9.0 limit		
						Max limit (ML)	No. of samples > ML	% Results > ML
Liquorice, root	conv.	Lead	159	43.6	1.23	5	1	0.6%
Liquorice, root	conv.	Mercury	150	0.03	n.c.	0.1	0	0.0%
Liquorice, root	orga.	Cadmium	77	0.145	n.c.	1	0	0.0%
Liquorice, root	orga.	Lead	79	2.71	n.c.	5	0	0.0%
Liquorice, root	orga.	Mercury	54	< 0.02	n.c.	0.1	0	0.0%
Lovage, root	conv.	Cadmium	57	0.56	0.38	1	0	0.0%
Lovage, root	conv.	Lead	54	3.59	2.78	5	0	0.0%
Lovage, root	conv.	Mercury	53	0.02	n.c.	0.1	0	0.0%
Mace, aril, Nutmeg	conv.	Cadmium	18	0.08	n.c.	1	0	0.0%
Mace, aril, Nutmeg	conv.	Lead	19	0.5	n.c.	5	0	0.0%
Mace, aril, Nutmeg	conv.	Mercury	18	< 0.02	n.c.	0.1	0	0.0%
Mallow, flowers	conv.	Cadmium	40	0.621	0.348	1	0	0.0%
Mallow, flowers	conv.	Lead	40	5.78	3.23	5	1	2.5%
Mallow, flowers	conv.	Mercury	28	0.03	n.c.	0.1	0	0.0%
Mallow, flowers	orga.	Cadmium	24	0.31	0.287	1	0	0.0%
Mallow, flowers	orga.	Lead	24	2.31	n.c.	5	0	0.0%
Mallow, flowers	orga.	Mercury	11	< 0.02	n.c.	0.1	0	0.0%
Mallow, herb	conv.	Cadmium	13	0.4	0.35	1	0	0.0%
Mallow, herb	conv.	Lead	13	5.75	n.c.	5	1	7.7%
Mallow, herb	conv.	Mercury	13	0.03	n.c.	0.1	0	0.0%
Mallow, leaves	conv.	Cadmium	81	0.55	0.34	1	0	0.0%
Mallow, leaves	conv.	Lead	80	11	3.78	5	5	6.3%
Mallow, leaves	conv.	Mercury	79	0.06	0.04	0.1	0	0.0%
Mallow, leaves	orga.	Cadmium	12	0.33	0.24	1	0	0.0%
Mallow, leaves	orga.	Lead	11	4.17	2.45	5	0	0.0%
Mallow, leaves	orga.	Mercury	12	0.02	n.c.	0.1	0	0.0%
Mandrake, root	conv.	Cadmium	12	< 0.07	n.c.	1	0	0.0%
Mandrake, root	conv.	Lead	12	0.5	n.c.	5	0	0.0%
Mandrake, root	conv.	Mercury	12	< 0.02	n.c.	0.1	0	0.0%
Marigold, flowers	conv.	Cadmium	71	0.22	0.19	1	0	0.0%
Marigold, flowers	conv.	Lead	71	7.94	1.66	5	1	1.4%
Marigold, flowers	conv.	Mercury	64	0.03	n.c.	0.1	0	0.0%
Marigold, flowers	orga.	Cadmium	64	0.303	0.295	1	0	0.0%
Marigold, flowers	orga.	Lead	66	9.23	1.61	5	1	1.5%
Marigold, flowers	orga.	Mercury	30	< 0.02	n.c.	0.1	0	0.0%
Marigold, herb	conv.	Cadmium	44	2.17	0.62	1	3	6.8%
Marigold, herb	conv.	Lead	44	1.54	n.c.	5	0	0.0%
Marigold, herb	conv.	Mercury	44	0.02	n.c.	0.1	0	0.0%
Marjoram, herb	conv.	Cadmium	47	0.3	0.23	1	0	0.0%
Marjoram, herb	conv.	Lead	46	4.22	2.44	5	0	0.0%
Marjoram, herb	conv.	Mercury	46	0.02	0.02	0.1	0	0.0%
Marjoram, herb	orga.	Cadmium	13	0.275	n.c.	1	0	0.0%
Marjoram, herb	orga.	Lead	13	1.61	1.1	5	0	0.0%
Marsh Trefoil, leaves	conv.	Cadmium	20	0.457	n.c.	1	0	0.0%
Marsh Trefoil, leaves	conv.	Lead	20	248	n.c.	5	1	5.0%
Marsh Trefoil, leaves	conv.	Mercury	16	0.02	n.c.	0.1	0	0.0%
Marshmallow, leaves	conv.	Cadmium	21	0.73	0.5	1	0	0.0%
Marshmallow, leaves	conv.	Lead	16	2.6	2.53	5	0	0.0%
Marshmallow, leaves	conv.	Mercury	15	0.02	n.c.	0.1	0	0.0%

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	Ph. Eur. 9.0 limit		
						Max limit (ML)	No. of samples > ML	% Results > ML
Marshmallow, leaves	orga.	Cadmium	22	0.33	0.2	1	0	0.0%
Marshmallow, leaves	orga.	Lead	22	7.57	1.46	5	1	4.5%
Marshmallow, leaves	orga.	Mercury	17	0.02	n.c.	0.1	0	0.0%
Marshmallow, root	conv.	Cadmium	104	0.85	0.71	1	0	0.0%
Marshmallow, root	conv.	Lead	102	25.9	1.7	5	1	1.0%
Marshmallow, root	conv.	Mercury	95	0.02	n.c.	0.1	0	0.0%
Marshmallow, root	orga.	Cadmium	15	0.47	0.35	1	0	0.0%
Marshmallow, root	orga.	Lead	15	1.43	1.39	5	0	0.0%
Marshmallow, root	orga.	Mercury	15	< 0.02	n.c.	0.1	0	0.0%
Maté, leaves	conv.	Cadmium	75	1.12	0.57	1	1	1.3%
Maté, leaves	conv.	Lead	63	0.77	n.c.	5	0	0.0%
Maté, leaves	conv.	Mercury	55	0.06	0.02	0.1	0	0.0%
Maté, leaves	orga.	Cadmium	38	0.71	0.425	1	0	0.0%
Maté, leaves	orga.	Lead	38	1.7	n.c.	5	0	0.0%
Maté, leaves	orga.	Mercury	23	0.02	n.c.	0.1	0	0.0%
Meadowsweet, flowers	conv.	Cadmium	18	0.81	0.67	1	0	0.0%
Meadowsweet, flowers	conv.	Lead	17	2.36	n.c.	5	0	0.0%
Meadowsweet, flowers	conv.	Mercury	17	< 0.02	n.c.	0.1	0	0.0%
Meadowsweet, herb	conv.	Cadmium	41	1.19	0.76	1	2	4.9%
Meadowsweet, herb	conv.	Lead	39	2.56	n.c.	5	0	0.0%
Meadowsweet, herb	conv.	Mercury	39	0.02	n.c.	0.1	0	0.0%
Medicinal Chinese Rhu, root	conv.	Cadmium	18	0.25	n.c.	1	0	0.0%
Medicinal Chinese Rhu, root	conv.	Lead	18	0.74	n.c.	5	0	0.0%
Medicinal Chinese Rhu, root	conv.	Mercury	18	< 0.02	n.c.	0.1	0	0.0%
Milk Thistle, fruit	conv.	Cadmium	56	0.8	0.441	1	0	0.0%
Milk Thistle, fruit	conv.	Lead	55	0.5	n.c.	5	0	0.0%
Milk Thistle, fruit	conv.	Mercury	34	< 0.02	n.c.	0.1	0	0.0%
Millet, seeds	conv.	Cadmium	29	0.1	n.c.	1	0	0.0%
Millet, seeds	conv.	Lead	32	0.5	n.c.	5	0	0.0%
Millet, seeds	conv.	Mercury	24	0.02	n.c.	0.1	0	0.0%
Mistletoe, herb	conv.	Cadmium	171	1.39	1.01	1	14	8.2%
Mistletoe, herb	conv.	Lead	168	16	1.07	5	1	0.6%
Mistletoe, herb	conv.	Mercury	155	0.04	0.03	0.1	0	0.0%
Mistletoe, herb	orga.	Cadmium	47	0.555	0.398	1	0	0.0%
Mistletoe, herb	orga.	Lead	47	1.6	n.c.	5	0	0.0%
Mistletoe, herb	orga.	Mercury	13	0.03	n.c.	0.1	0	0.0%
Motherwort, herb	conv.	Cadmium	13	0.1	n.c.	1	0	0.0%
Motherwort, herb	conv.	Lead	13	1.17	n.c.	5	0	0.0%
Motherwort, herb	conv.	Mercury	12	0.03	n.c.	0.1	0	0.0%
Mullein, flowers	conv.	Cadmium	16	< 0.07	n.c.	1	0	0.0%
Mullein, flowers	conv.	Lead	16	1.21	1.11	5	0	0.0%
Mullein, flowers	conv.	Mercury	12	< 0.02	n.c.	0.1	0	0.0%
Nana mint, leaves	orga.	Cadmium	39	0.091	n.c.	1	0	0.0%
Nana mint, leaves	orga.	Lead	40	1.9	1.34	5	0	0.0%
Oak, bark	conv.	Cadmium	52	0.52	0.45	1	0	0.0%
Oak, bark	conv.	Lead	57	6.7	2.53	5	3	5.3%
Oak, bark	conv.	Mercury	50	0.03	n.c.	0.1	0	0.0%

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	Ph. Eur. 9.0 limit		
						Max limit (ML)	No. of samples > ML	% Results > ML
Oats, straw, green	conv.	Cadmium	23	0.2	n.c.	1	0	0.0%
Oats, straw, green	conv.	Lead	22	1.79	n.c.	5	0	0.0%
Oats, straw, green	conv.	Mercury	20	0.06	n.c.	0.1	0	0.0%
Oats, straw, green	orga.	Cadmium	18	0.082	n.c.	1	0	0.0%
Oats, straw, green	orga.	Lead	20	0.5	n.c.	5	0	0.0%
Oats, straw, green	orga.	Mercury	14	< 0.02	n.c.	0.1	0	0.0%
Olive, leaves	conv.	Cadmium	51	< 0.07	n.c.	1	0	0.0%
Olive, leaves	conv.	Lead	51	1.72	1.39	5	0	0.0%
Olive, leaves	conv.	Mercury	48	0.07	0.05	0.1	0	0.0%
Onion, bulb	conv.	Cadmium	14	< 0.07	n.c.	1	0	0.0%
Onion, bulb	conv.	Lead	14	0.5	n.c.	5	0	0.0%
Onion, bulb	conv.	Mercury	14	< 0.02	n.c.	0.1	0	0.0%
Paeony, flowers	conv.	Cadmium	11	0.253	n.c.	1	0	0.0%
Paeony, flowers	conv.	Lead	12	0.562	n.c.	5	0	0.0%
Parsley, root	conv.	Cadmium	20	0.538	0.523	1	0	0.0%
Parsley, root	conv.	Lead	20	0.5	n.c.	5	0	0.0%
Parsley, root	conv.	Mercury	15	< 0.02	n.c.	0.1	0	0.0%
Passion Flower, herb	conv.	Cadmium	107	0.4	0.261	1	0	0.0%
Passion Flower, herb	conv.	Lead	105	3.4	1.33	5	0	0.0%
Passion Flower, herb	conv.	Mercury	85	0.05	0.036	0.1	0	0.0%
Passion Flower, herb	orga.	Cadmium	27	0.199	0.143	1	0	0.0%
Passion Flower, herb	orga.	Lead	27	1.71	1.66	5	0	0.0%
Passion Flower, herb	orga.	Mercury	15	0.02	n.c.	0.1	0	0.0%
Pelargonium, root	conv.	Cadmium	10	0.1	n.c.	1	0	0.0%
Peppermint, leaves	conv.	Cadmium	75	0.55	0.21	1	0	0.0%
Peppermint, leaves	conv.	Lead	84	1.35	1.26	5	0	0.0%
Peppermint, leaves	conv.	Mercury	59	0.04	0.02	0.1	0	0.0%
Peppermint, leaves	orga.	Cadmium	262	0.983	0.267	1	0	0.0%
Peppermint, leaves	orga.	Lead	267	10.1	1.42	5	1	0.4%
Peppermint, leaves	orga.	Mercury	223	0.04	0.03	0.1	0	0.0%
Peruvian bark, bark	conv.	Cadmium	14	1.92	1.78	1	6	42.9%
Peruvian bark, bark	conv.	Lead	14	0.5	n.c.	5	0	0.0%
Peruvian bark, bark	conv.	Mercury	14	0.02	n.c.	0.1	0	0.0%
Pu Erh Tea, leaves	conv.	Cadmium	40	0.09	0.08	1	0	0.0%
Pu Erh Tea, leaves	conv.	Lead	72	12.4	3.65	5	5	6.9%
Pu Erh Tea, leaves	conv.	Mercury	27	0.08	0.03	0.1	0	0.0%
Pumpkin, seeds	conv.	Cadmium	96	0.23	n.c.	1	0	0.0%
Pumpkin, seeds	conv.	Lead	91	0.95	n.c.	5	0	0.0%
Pumpkin, seeds	conv.	Mercury	86	0.1	0.02	0.1	0	0.0%
Pumpkin, seeds	orga.	Cadmium	12	< 0.07	n.c.	1	0	0.0%
Pumpkin, seeds	orga.	Lead	12	0.5	n.c.	5	0	0.0%
Purlanes, herb	conv.	Cadmium	19	0.3	0.22	1	0	0.0%
Purlanes, herb	conv.	Lead	19	2.19	1.46	5	0	0.0%
Raspberry, fruit	conv.	Cadmium	22	0.26	0.2	1	0	0.0%
Raspberry, fruit	conv.	Lead	22	3.08	n.c.	5	0	0.0%
Raspberry, fruit	conv.	Mercury	22	< 0.02	n.c.	0.1	0	0.0%
Raspberry, leaves	conv.	Cadmium	21	0.421	0.28	1	0	0.0%
Raspberry, leaves	conv.	Lead	18	1.9	1.34	5	0	0.0%
Raspberry, leaves	conv.	Mercury	11	0.03	n.c.	0.1	0	0.0%

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	Ph. Eur. 9.0 limit		
						Max limit (ML)	No. of samples > ML	% Results > ML
Raspberry, leaves	orga.	Cadmium	75	0.573	0.351	1	0	0.0%
Raspberry, leaves	orga.	Lead	75	16.6	1.6	5	1	1.3%
Raspberry, leaves	orga.	Mercury	41	0.03	0.025	0.1	0	0.0%
Rauvolfia, root	conv.	Cadmium	14	0.2	n.c.	1	0	0.0%
Rauvolfia, root	conv.	Lead	14	0.5	n.c.	5	0	0.0%
Rauvolfia, root	conv.	Mercury	14	0.02	n.c.	0.1	0	0.0%
Red Clover, flowers	conv.	Cadmium	19	< 0.07	n.c.	1	0	0.0%
Red Clover, flowers	conv.	Lead	19	1.47	n.c.	5	0	0.0%
Red Clover, flowers	conv.	Mercury	19	< 0.02	n.c.	0.1	0	0.0%
Red vine leaves, leaves	conv.	Cadmium	170	< 0.07	n.c.	1	0	0.0%
Red vine leaves, leaves	conv.	Lead	169	3.3	1.22	5	0	0.0%
Red vine leaves, leaves	conv.	Mercury	169	0.08	0.04	0.1	0	0.0%
Reindeer lichen	conv.	Cadmium	19	0.19	0.12	1	0	0.0%
Reindeer lichen	conv.	Lead	19	3.51	2.23	5	0	0.0%
Reindeer lichen	conv.	Mercury	19	0.04	0.04	0.1	0	0.0%
Rest Harrow, root	conv.	Cadmium	38	0.54	0.17	1	0	0.0%
Rest Harrow, root	conv.	Lead	36	2.29	1.82	5	0	0.0%
Rest Harrow, root	conv.	Mercury	34	0.02	n.c.	0.1	0	0.0%
Rhatany, root	conv.	Cadmium	40	0.2	0.17	1	0	0.0%
Rhatany, root	conv.	Lead	40	0.64	n.c.	5	0	0.0%
Rhatany, root	conv.	Mercury	40	< 0.02	n.c.	0.1	0	0.0%
Ribwort, herb	conv.	Cadmium	264	1.05	0.4	1	1	0.4%
Ribwort, herb	conv.	Lead	264	6	1.71	5	1	0.4%
Ribwort, herb	conv.	Mercury	263	0.04	0.02	0.1	0	0.0%
Ribwort, herb	orga.	Cadmium	41	1	0.653	1	0	0.0%
Ribwort, herb	orga.	Lead	39	1.81	1.65	5	0	0.0%
Ribwort, herb	orga.	Mercury	11	< 0.02	n.c.	0.1	0	0.0%
Rock rose, herb	conv.	Cadmium	23	1.2	0.87	1	1	4.3%
Rock rose, herb	conv.	Lead	15	1.46	n.c.	5	0	0.0%
Rock rose, herb	orga.	Cadmium	23	1.41	0.589	1	1	4.3%
Rock rose, herb	orga.	Lead	23	1.55	1	5	0	0.0%
Roman Camomile, flowers	conv.	Cadmium	11	0.529	0.505	1	0	0.0%
Roman Camomile, flowers	conv.	Lead	11	0.604	n.c.	5	0	0.0%
Rooibos, leaves	orga.	Cadmium	79	0.096	0.08	1	0	0.0%
Rooibos, leaves	orga.	Lead	80	1.7	1.5	5	0	0.0%
Rooibos, leaves	orga.	Mercury	63	0.03	n.c.	0.1	0	0.0%
Rose Hip	conv.	Cadmium	10	< 0.07	n.c.	1	0	0.0%
Rose Hip	conv.	Lead	10	0.5	n.c.	5	0	0.0%
Rose Hip	orga.	Cadmium	44	< 0.07	n.c.	1	0	0.0%
Rose Hip	orga.	Lead	44	0.69	n.c.	5	0	0.0%
Rose Hip	orga.	Mercury	44	< 0.02	n.c.	0.1	0	0.0%
Rose Hip, shells	orga.	Cadmium	109	< 0.07	n.c.	1	0	0.0%
Rose Hip, shells	orga.	Lead	112	0.54	n.c.	5	0	0.0%
Rose Hip, shells	orga.	Mercury	79	0.02	n.c.	0.1	0	0.0%
Rose, petals	conv.	Cadmium	18	< 0.07	n.c.	1	0	0.0%
Rose, petals	conv.	Lead	18	1.75	n.c.	5	0	0.0%
Rose, petals	conv.	Mercury	18	< 0.02	n.c.	0.1	0	0.0%
Rose, petals	orga.	Cadmium	23	< 0.07	n.c.	1	0	0.0%
Rose, petals	orga.	Lead	23	3.81	n.c.	5	0	0.0%

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	Ph. Eur. 9.0 limit		
						Max limit (ML)	No. of samples > ML	% Results > ML
Rosemary leaves	conv.	Cadmium	17	0.54	n.c.	1	0	0.0%
Rosemary leaves	conv.	Lead	16	4.14	n.c.	5	0	0.0%
Rosemary leaves	conv.	Mercury	15	0.08	0.04	0.1	0	0.0%
Rosemary leaves	orga.	Cadmium	31	< 0.07	n.c.	1	0	0.0%
Rosemary leaves	orga.	Lead	32	1.5	1.43	5	0	0.0%
Rosemary leaves	orga.	Mercury	18	0.07	0.06	0.1	0	0.0%
Roseroot, roots	conv.	Cadmium	12	0.46	0.39	1	0	0.0%
Roseroot, roots	conv.	Lead	12	1.33	1.21	5	0	0.0%
Roseroot, roots	conv.	Mercury	12	< 0.02	n.c.	0.1	0	0.0%
Safflower, flowers	conv.	Cadmium	31	0.08	n.c.	1	0	0.0%
Safflower, flowers	conv.	Lead	32	132	2.29	5	2	6.3%
Safflower, flowers	conv.	Mercury	31	0.17	n.c.	0.1	1	3.2%
Sage, leaves	conv.	Cadmium	99	0.12	n.c.	1	0	0.0%
Sage, leaves	conv.	Lead	99	3.57	1.37	5	0	0.0%
Sage, leaves	conv.	Mercury	98	0.04	0.03	0.1	0	0.0%
Sage, leaves	orga.	Cadmium	112	0.157	n.c.	1	0	0.0%
Sage, leaves	orga.	Lead	117	4.48	1.68	5	0	0.0%
Sage, leaves	orga.	Mercury	78	0.05	0.02	0.1	0	0.0%
Sandy Everlasting, flowers	conv.	Cadmium	11	0.62	0.6	1	0	0.0%
Sandy Everlasting, flowers	conv.	Lead	11	0.5	n.c.	5	0	0.0%
Saw Palmetto, fruit	conv.	Cadmium	46	< 0.07	n.c.	1	0	0.0%
Saw Palmetto, fruit	conv.	Lead	46	0.5	n.c.	5	0	0.0%
Saw Palmetto, fruit	conv.	Mercury	46	< 0.02	n.c.	0.1	0	0.0%
Scarlet pimpernel, herb	conv.	Cadmium	10	< 0.07	n.c.	1	0	0.0%
Scarlet pimpernel, herb	conv.	Lead	10	1.58	n.c.	5	0	0.0%
Scarlet pimpernel, herb	conv.	Mercury	10	0.02	n.c.	0.1	0	0.0%
Scotch Pine, cones	conv.	Cadmium	12	0.19	0.11	1	0	0.0%
Sea Buckthorn, fruit	orga.	Cadmium	16	< 0.07	n.c.	1	0	0.0%
Sea Buckthorn, fruit	orga.	Lead	17	0.5	n.c.	5	0	0.0%
Seaweed	conv.	Cadmium	18	7.73	5.71	1	17	94.4%
Seaweed	conv.	Lead	18	3.05	n.c.	5	0	0.0%
Seaweed	conv.	Mercury	13	< 0.02	n.c.	0.1	0	0.0%
Senna, leaves (C. acutifolia)	conv.	Cadmium	178	< 0.07	n.c.	1	0	0.0%
Senna, leaves (C. acutifolia)	conv.	Lead	179	1.54	0.95	5	0	0.0%
Senna, leaves (C. acutifolia)	conv.	Mercury	178	0.05	0.03	0.1	0	0.0%
Senna, leaves (C. acutifolia)	orga.	Cadmium	25	< 0.07	n.c.	1	0	0.0%
Senna, leaves (C. acutifolia)	orga.	Lead	25	0.74	0.74	5	0	0.0%
Senna, leaves (C. acutifolia)	orga.	Mercury	25	0.02	n.c.	0.1	0	0.0%
Senna, leaves (C. angustifolia)	conv.	Cadmium	23	< 0.07	n.c.	1	0	0.0%
Senna, leaves (C. angustifolia)	conv.	Lead	24	0.656	n.c.	5	0	0.0%
Senna, Pods (C. acutifolia)	conv.	Cadmium	38	< 0.07	n.c.	1	0	0.0%

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	Ph. Eur. 9.0 limit		
						Max limit (ML)	No. of samples > ML	% Results > ML
Senna, Pods (C. acutifolia)	conv.	Lead	38	1.97	n.c.	5	0	0.0%
Senna, Pods (C. acutifolia)	conv.	Mercury	38	0.02	n.c.	0.1	0	0.0%
Senna, Pods (C. angustifolia)	conv.	Cadmium	58	< 0.07	n.c.	1	0	0.0%
Senna, Pods (C. angustifolia)	conv.	Lead	61	0.94	n.c.	5	0	0.0%
Senna, Pods (C. angustifolia)	conv.	Mercury	55	0.13	n.c.	0.1	1	1.8%
Sheep's bane, herb	conv.	Cadmium	52	1.4	0.68	1	2	3.8%
Sheep's bane, herb	conv.	Lead	52	3.97	1.46	5	0	0.0%
Sheep's bane, herb	conv.	Mercury	49	0.04	n.c.	0.1	0	0.0%
Shepherd's purse, herb	conv.	Cadmium	18	0.23	0.22	1	0	0.0%
Shepherd's purse, herb	conv.	Lead	18	0.95	n.c.	5	0	0.0%
Shepherd's purse, herb	conv.	Mercury	18	0.03	n.c.	0.1	0	0.0%
Siberian ginseng, root	conv.	Cadmium	25	0.3	0.21	1	0	0.0%
Siberian ginseng, root	conv.	Lead	25	7.54	1.9	5	1	4.0%
Siberian ginseng, root	conv.	Mercury	18	< 0.02	n.c.	0.1	0	0.0%
Silver Lime, flowers	conv.	Cadmium	160	0.43	0.14	1	0	0.0%
Silver Lime, flowers	conv.	Lead	161	24	3.14	5	6	3.7%
Silver Lime, flowers	conv.	Mercury	160	0.06	0.05	0.1	0	0.0%
Silver Lime, flowers	orga.	Cadmium	33	0.14	0.13	1	0	0.0%
Silver Lime, flowers	orga.	Lead	33	7.86	3.39	5	1	3.0%
Silver Lime, flowers	orga.	Mercury	33	0.03	n.c.	0.1	0	0.0%
Sloe, flowers	conv.	Cadmium	26	0.09	n.c.	1	0	0.0%
Sloe, flowers	conv.	Lead	21	3.83	2.89	5	0	0.0%
Sloe, flowers	conv.	Mercury	20	0.02	n.c.	0.1	0	0.0%
Spinach, leaves	conv.	Cadmium	30	1.75	1.53	1	14	46.7%
Spinach, leaves	conv.	Lead	30	1.03	0.807	5	0	0.0%
Spinach, leaves	orga.	Cadmium	12	0.889	0.8	1	0	0.0%
Spinach, leaves	orga.	Lead	12	0.775	n.c.	5	0	0.0%
St John's wort, herb	conv.	Cadmium	330	3.18	0.93	1	25	7.6%
St John's wort, herb	conv.	Lead	258	17.5	4.1	5	5	1.9%
St John's wort, herb	conv.	Mercury	208	0.1	n.c.	0.1	0	0.0%
St John's wort, herb	orga.	Cadmium	36	1.36	0.809	1	2	5.6%
St John's wort, herb	orga.	Lead	36	0.673	n.c.	5	0	0.0%
Star Anise, fruit	conv.	Cadmium	19	< 0.07	n.c.	1	0	0.0%
Star Anise, fruit	conv.	Lead	19	1.13	n.c.	5	0	0.0%
Star Anise, fruit	conv.	Mercury	18	0.03	n.c.	0.1	0	0.0%
Star Anise, fruit	orga.	Cadmium	13	< 0.07	n.c.	1	0	0.0%
Star Anise, fruit	orga.	Lead	13	1.18	n.c.	5	0	0.0%
Star Anise, fruit	orga.	Mercury	13	0.04	0.02	0.1	0	0.0%
Stinging Nettle	conv.	Cadmium	32	0.2	n.c.	1	0	0.0%
Stinging Nettle	conv.	Lead	32	5.45	3.6	5	1	3.1%
Stinging Nettle	conv.	Mercury	14	0.02	n.c.	0.1	0	0.0%
Stinging Nettle	orga.	Cadmium	113	0.108	n.c.	1	0	0.0%
Stinging Nettle	orga.	Lead	121	2.86	1.8	5	0	0.0%
Stinging Nettle	orga.	Mercury	16	< 0.02	n.c.	0.1	0	0.0%
Stinging Nettle, Dwarf Nettle, herb	conv.	Cadmium	113	0.16	0.14	1	0	0.0%

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	Ph. Eur. 9.0 limit		
						Max limit (ML)	No. of samples > ML	% Results > ML
Stinging Nettle, Dwarf Nettle, herb	conv.	Lead	119	510	14.8	5	19	16.0%
Stinging Nettle, Dwarf Nettle, herb	conv.	Mercury	112	0.03	0.02	0.1	0	0.0%
Stinging Nettle, Dwarf Nettle, herb	orga.	Cadmium	85	0.09	n.c.	1	0	0.0%
Stinging Nettle, Dwarf Nettle, herb	orga.	Lead	86	7.62	3.29	5	4	4.7%
Stinging Nettle, Dwarf Nettle, herb	orga.	Mercury	85	0.07	0.03	0.1	0	0.0%
Stinging Nettle, Dwarf Nettle, root	conv.	Cadmium	48	0.22	0.15	1	0	0.0%
Stinging Nettle, Dwarf Nettle, root	conv.	Lead	50	19.3	5.58	7	4	8.0%
Stinging Nettle, Dwarf Nettle, root	conv.	Mercury	43	0.05	0.04	0.1	0	0.0%
Strawberry, fruit	conv.	Cadmium	22	0.9	n.c.	1	0	0.0%
Strawberry, fruit	conv.	Lead	22	3.54	n.c.	5	0	0.0%
Strawberry, fruit	conv.	Mercury	22	< 0.02	n.c.	0.1	0	0.0%
Strawberry, leaves	conv.	Cadmium	82	0.21	n.c.	1	0	0.0%
Strawberry, leaves	conv.	Lead	81	2.7	1.08	5	0	0.0%
Strawberry, leaves	conv.	Mercury	81	0.02	n.c.	0.1	0	0.0%
Strawberry, leaves	orga.	Cadmium	35	0.23	n.c.	1	0	0.0%
Strawberry, leaves	orga.	Lead	37	6.53	3.01	5	1	2.7%
Strawberry, leaves	orga.	Mercury	28	0.02	n.c.	0.1	0	0.0%
Strophantus, seeds	conv.	Cadmium	26	0.07	n.c.	1	0	0.0%
Strophantus, seeds	conv.	Lead	26	0.5	n.c.	5	0	0.0%
Strophantus, seeds	conv.	Mercury	26	< 0.02	n.c.	0.1	0	0.0%
Strychnine, seeds	conv.	Cadmium	10	< 0.07	n.c.	1	0	0.0%
Strychnine, seeds	conv.	Lead	10	2.91	n.c.	5	0	0.0%
Strychnine, seeds	conv.	Mercury	10	0.03	n.c.	0.1	0	0.0%
Sundew, herb	conv.	Cadmium	12	2.96	n.c.	1	2	16.7%
Sundew, herb	conv.	Lead	13	23.4	14.6	5	4	30.8%
Sundew, herb	conv.	Mercury	11	0.05	n.c.	0.1	0	0.0%
Sunflower, flowers	orga.	Cadmium	21	0.185	0.11	1	0	0.0%
Sunflower, flowers	orga.	Lead	21	0.76	n.c.	5	0	0.0%
Sunflower, flowers	orga.	Mercury	15	< 0.02	n.c.	0.1	0	0.0%
Sweet Chestnut, leaves	conv.	Cadmium	10	0.18	n.c.	1	0	0.0%
Sweet Chestnut, leaves	conv.	Lead	10	0.69	n.c.	5	0	0.0%
Sweet Chestnut, leaves	conv.	Mercury	10	0.02	n.c.	0.1	0	0.0%
Sweet Orange, leaves	orga.	Cadmium	13	< 0.07	n.c.	1	0	0.0%
Sweet Orange, leaves	orga.	Lead	13	1.12	n.c.	5	0	0.0%
Sweet Orange, leaves	orga.	Mercury	14	0.07	0.07	0.1	0	0.0%
Sweet Orange, peel	conv.	Cadmium	40	< 0.07	n.c.	1	0	0.0%
Sweet Orange, peel	conv.	Lead	40	0.52	n.c.	5	0	0.0%
Sweet Orange, peel	conv.	Mercury	40	< 0.02	n.c.	0.1	0	0.0%
Sweet Orange, peel	orga.	Cadmium	54	< 0.07	n.c.	1	0	0.0%
Sweet Orange, peel	orga.	Lead	55	0.5	n.c.	5	0	0.0%
Sweet Orange, peel	orga.	Mercury	36	0.02	n.c.	0.1	0	0.0%
Sweet Sedge, rhizome	conv.	Cadmium	40	0.12	0.1	1	0	0.0%
Sweet Sedge, rhizome	conv.	Lead	38	5.86	n.c.	5	1	2.6%
Sweet Sedge, rhizome	conv.	Mercury	37	0.02	n.c.	0.1	0	0.0%

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	Ph. Eur. 9.0 limit		
						Max limit (ML)	No. of samples > ML	% Results > ML
Tea (Camellia sinensis)	conv.	Cadmium	125	0.14	0.1	1	0	0.0%
Tea (Camellia sinensis)	conv.	Lead	265	6.52	2.44	5	1	0.4%
Tea (Camellia sinensis)	conv.	Mercury	117	0.08	0.06	0.1	0	0.0%
Tea (Camellia sinensis)	orga.	Cadmium	107	0.159	0.127	1	0	0.0%
Tea (Camellia sinensis)	orga.	Lead	107	4.05	2.67	5	0	0.0%
Tea (Camellia sinensis)	orga.	Mercury	26	< 0.02	n.c.	0.1	0	0.0%
Thyme, herb	conv.	Cadmium	305	1.84	0.53	1	5	1.6%
Thyme, herb	conv.	Lead	294	3.5	1.94	5	0	0.0%
Thyme, herb	conv.	Mercury	285	0.08	0.04	0.1	0	0.0%
Thyme, herb	orga.	Cadmium	111	0.675	0.293	1	0	0.0%
Thyme, herb	orga.	Lead	113	3.68	2.29	5	0	0.0%
Thyme, herb	orga.	Mercury	63	0.05	0.04	0.1	0	0.0%
Toothpick, fruit	conv.	Cadmium	13	0.07	n.c.	1	0	0.0%
Toothpick, fruit	conv.	Lead	13	0.5	n.c.	5	0	0.0%
Toothpick, fruit	conv.	Mercury	13	0.02	n.c.	0.1	0	0.0%
Tormentil, rhizome	conv.	Cadmium	39	2.28	2.15	2	5	12.8%
Tormentil, rhizome	conv.	Lead	31	14.3	4.8	5	2	6.5%
Tormentil, rhizome	conv.	Mercury	31	0.02	n.c.	0.1	0	0.0%
Turmeric, rhizome	conv.	Cadmium	17	0.65	n.c.	1	0	0.0%
Turmeric, rhizome	conv.	Lead	17	2.83	n.c.	5	0	0.0%
Uzara, root	conv.	Cadmium	10	< 0.07	n.c.	1	0	0.0%
Uzara, root	conv.	Lead	10	2.15	n.c.	5	0	0.0%
Uzara, root	conv.	Mercury	10	< 0.02	n.c.	0.1	0	0.0%
Valerian, root	conv.	Cadmium	382	0.39	0.21	1	0	0.0%
Valerian, root	conv.	Lead	389	10.7	3.52	5	17	4.4%
Valerian, root	conv.	Mercury	347	0.26	0.1	0.1	1	0.3%
Valerian, root	orga.	Cadmium	23	0.172	0.17	1	0	0.0%
Valerian, root	orga.	Lead	24	2.25	1.71	5	0	0.0%
Valerian, root	orga.	Mercury	12	0.02	n.c.	0.1	0	0.0%
Vanilla	conv.	Cadmium	17	0.22	n.c.	1	0	0.0%
Vanilla	conv.	Lead	17	0.5	n.c.	5	0	0.0%
Vanilla	conv.	Mercury	15	0.02	0.02	0.1	0	0.0%
Vervain, herb	conv.	Cadmium	20	0.12	n.c.	1	0	0.0%
Vervain, herb	conv.	Lead	20	2.32	n.c.	5	0	0.0%
Vervain, herb	conv.	Mercury	19	0.02	n.c.	0.1	0	0.0%
Walnut, leaves	orga.	Cadmium	12	< 0.07	n.c.	1	0	0.0%
Walnut, leaves	orga.	Lead	13	0.765	n.c.	5	0	0.0%
Watercress, herb	conv.	Cadmium	65	1.72	1.08	1	9	13.8%
Watercress, herb	conv.	Lead	65	10.6	7.44	5	10	15.4%
Watercress, herb	conv.	Mercury	57	0.03	n.c.	0.1	0	0.0%
Watercress, herb	orga.	Cadmium	14	1.01	0.774	1	1	7.1%
Watercress, herb	orga.	Lead	14	4.5	2.55	5	0	0.0%
White Mustard, seeds	conv.	Cadmium	10	0.337	0.236	1	0	0.0%
White Mustard, seeds	conv.	Lead	10	0.5	n.c.	5	0	0.0%
Willow herb, herb	conv.	Cadmium	15	0.106	n.c.	1	0	0.0%
Willow herb, herb	conv.	Lead	15	1.65	n.c.	5	0	0.0%
Willow herb, herb	conv.	Mercury	11	< 0.02	n.c.	0.1	0	0.0%
Willow herb, herb	orga.	Lead	10	0.914	n.c.	5	0	0.0%
Willow, bark	conv.	Cadmium	155	8.21	2.74	2	30	19.4%

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	Ph. Eur. 9.0 limit		
						Max limit (ML)	No. of samples > ML	% Results > ML
Willow, bark	conv.	Lead	61	4.66	1.92	5	0	0.0%
Willow, bark	conv.	Mercury	53	0.02	0.02	0.1	0	0.0%
Willow, leaves	conv.	Cadmium	32	2.13	1.57	1	14	43.8%
Willow, leaves	conv.	Lead	30	1.11	n.c.	5	0	0.0%
Willow, leaves	conv.	Mercury	30	0.02	n.c.	0.1	0	0.0%
Witch Hazel, bark	conv.	Cadmium	15	0.08	n.c.	1	0	0.0%
Witch Hazel, bark	conv.	Lead	14	2.13	1.5	5	0	0.0%
Witch Hazel, bark	conv.	Mercury	14	< 0.02	n.c.	0.1	0	0.0%
Witch Hazel, leaves	conv.	Cadmium	39	0.08	n.c.	1	0	0.0%
Witch Hazel, leaves	conv.	Lead	40	1.02	n.c.	5	0	0.0%
Witch Hazel, leaves	conv.	Mercury	39	0.03	0.03	0.1	0	0.0%
Yarrow, flowers	conv.	Cadmium	18	0.85	0.85	1	0	0.0%
Yarrow, flowers	conv.	Lead	16	0.5	n.c.	5	0	0.0%
Yarrow, flowers	conv.	Mercury	16	0.03	n.c.	0.1	0	0.0%
Yarrow, flowers	orga.	Cadmium	24	0.872	0.428	1	0	0.0%
Yarrow, flowers	orga.	Lead	24	4.09	n.c.	5	0	0.0%
Yarrow, flowers	orga.	Mercury	10	< 0.02	n.c.	0.1	0	0.0%
Yarrow, herb	conv.	Cadmium	52	1.17	0.93	1	3	5.8%
Yarrow, herb	conv.	Lead	51	0.699	n.c.	5	0	0.0%
Yarrow, herb	conv.	Mercury	40	0.02	n.c.	0.1	0	0.0%
Yarrow, herb	orga.	Cadmium	72	0.656	0.447	1	0	0.0%
Yarrow, herb	orga.	Lead	72	5.57	n.c.	5	1	1.4%
Yarrow, herb	orga.	Mercury	29	< 0.02	n.c.	0.1	0	0.0%
Yellow Gentian, root	conv.	Cadmium	63	0.56	0.37	1	0	0.0%
Yellow Gentian, root	conv.	Lead	61	4.4	2.2	5	0	0.0%
Yellow Gentian, root	conv.	Mercury	55	0.03	n.c.	0.1	0	0.0%

Appendix 2. *Results for arsenic, cobalt, nickel and vanadium in herbal drugs*

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	ICH Q3D Limit (10 g)		
						Max limit (ML)	No. of samples > ML	% Results > ML
Alder Buckthorn, bark	conv.	Arsenic	24	2.21	n.c.	1.5	1	4.2%
Alder Buckthorn, bark	conv.	Cobalt	17	0.198	n.c.	5	0	0.0%
Alder Buckthorn, bark	conv.	Nickel	18	0.91	0.864	20	0	0.0%
Alder Buckthorn, bark	conv.	Vanadium	17	0.26	0.259	10	0	0.0%
Angelica, root	conv.	Arsenic	16	0.7	n.c.	1.5	0	0.0%
Angelica, root	conv.	Cobalt	20	0.719	0.474	5	0	0.0%
Angelica, root	conv.	Nickel	30	26	7.75	20	1	3.3%
Angelica, root	conv.	Vanadium	20	2.615	1.909	10	0	0.0%
Aniseed	conv.	Cobalt	10	0.401	0.329	5	0	0.0%
Aniseed	conv.	Nickel	11	3.63	3.17	20	0	0.0%
Aniseed	conv.	Vanadium	10	0.936	0	10	0	0.0%
Aniseed	orga.	Cobalt	35	0.406	0.384	5	0	0.0%
Aniseed	orga.	Nickel	35	2.19	1.78	20	0	0.0%
Aniseed	orga.	Vanadium	35	1.032	0.521	10	0	0.0%
Apple, fruit	orga.	Cobalt	17	0.156	n.c.	5	0	0.0%
Apple, fruit	orga.	Nickel	17	1.72	1.68	20	0	0.0%
Apple, fruit	orga.	Vanadium	17	0.418	0.319	10	0	0.0%
Arnica, flowers	conv.	Cobalt	16	0.522	0.429	5	0	0.0%
Arnica, flowers	conv.	Nickel	18	14.8	14.4	20	0	0.0%
Arnica, flowers	conv.	Vanadium	16	0.692	0.357	10	0	0.0%
Bearberry, leaves	orga.	Cobalt	14	0.37	n.c.	5	0	0.0%
Bearberry, leaves	orga.	Nickel	13	3.3	2.89	20	0	0.0%
Bearberry, leaves	orga.	Vanadium	14	1.179	1.178	10	0	0.0%
Bilberry, fruit	orga.	Cobalt	27	0.365	n.c.	5	0	0.0%
Bilberry, fruit	orga.	Nickel	27	4.44	2.07	20	0	0.0%
Bilberry, fruit	orga.	Vanadium	27	0.968	0.807	10	0	0.0%
Birch, leaves	conv.	Arsenic	13	< 0.7	n.c.	1.5	0	0.0%
Birch, leaves	orga.	Cobalt	56	1.12	0.664	5	0	0.0%
Birch, leaves	orga.	Nickel	56	9.48	4.94	20	0	0.0%
Birch, leaves	orga.	Vanadium	56	0.267	0	10	0	0.0%
Blackberry, leaves	orga.	Cobalt	38	8.8	0.858	5	1	2.6%
Blackberry, leaves	orga.	Nickel	38	135	9.17	20	1	2.6%
Blackberry, leaves	orga.	Vanadium	38	4.369	1.278	10	0	0.0%
Blackcurrant, fruit	orga.	Cobalt	20	0.254	n.c.	5	0	0.0%
Blackcurrant, fruit	orga.	Nickel	20	3.53	1.18	20	0	0.0%
Blackcurrant, fruit	orga.	Vanadium	20	0.629	0	10	0	0.0%
Bladderwrack	conv.	Arsenic	32	79.6	76	1.5	32	100.0%
Blond Psyllium, seeds	conv.	Cobalt	17	0.234	0.203	5	0	0.0%
Blond Psyllium, seeds	conv.	Nickel	16	1.78	0.544	20	0	0.0%
Blond Psyllium, seeds	conv.	Vanadium	17	0.454	0.22	10	0	0.0%
Blond Psyllium, seeds	orga.	Cobalt	23	0.312	n.c.	5	0	0.0%
Blond Psyllium, seeds	orga.	Nickel	22	2.89	2.12	20	0	0.0%
Blond Psyllium, seeds	orga.	Vanadium	23	0.634	0.33	10	0	0.0%
Camomile, flowers	conv.	Arsenic	35	< 0.7	n.c.	1.5	0	0.0%
Camomile, flowers	conv.	Nickel	21	7.8	3.39	20	0	0.0%
Camomile, flowers	orga.	Cobalt	65	1.17	0.518	5	0	0.0%
Camomile, flowers	orga.	Nickel	65	9.28	4.53	20	0	0.0%
Camomile, flowers	orga.	Vanadium	65	5.06	1.773	10	0	0.0%

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	ICH Q3D Limit (10 g)		
						Max limit (ML)	No. of samples > ML	% Results > ML
Caraway, seeds	orga.	Cobalt	28	0.239	n.c.	5	0	0.0%
Caraway, seeds	orga.	Nickel	28	6.95	6.04	20	0	0.0%
Caraway, seeds	orga.	Vanadium	28	0.218	0	10	0	0.0%
Cardamom, fruit	orga.	Cobalt	10	0.494	0.268	5	0	0.0%
Cardamom, fruit	orga.	Nickel	10	7.23	2.76	20	0	0.0%
Cardamom, fruit	orga.	Vanadium	10	0.253	0	10	0	0.0%
Carrot	conv.	Cobalt	18	0.104	n.c.	5	0	0.0%
Carrot	conv.	Nickel	18	0.939	0.913	20	0	0.0%
Carrot	conv.	Vanadium	18	0	0	10	0	0.0%
Carrot	orga.	Cobalt	15	< 0.1	n.c.	5	0	0.0%
Carrot	orga.	Nickel	15	1.06	0.964	20	0	0.0%
Carrot	orga.	Vanadium	15	0.172	0	10	0	0.0%
Centaury, herb	conv.	Cobalt	15	0.634	0.599	5	0	0.0%
Centaury, herb	conv.	Nickel	15	8.2	7.45	20	0	0.0%
Centaury, herb	conv.	Vanadium	15	1.376	1.301	10	0	0.0%
Chondria	conv.	Arsenic	15	13.3	11.9	1.5	15	100.0%
Chondria	conv.	Cobalt	16	0.493	0.406	5	0	0.0%
Chondria	conv.	Nickel	16	9.99	8.46	20	0	0.0%
Chondria	conv.	Vanadium	16	13.488	10.823	10	3	18.8%
Cinnamon, bark	conv.	Cobalt	21	0.418	0.208	5	0	0.0%
Cinnamon, bark	conv.	Nickel	21	1.29	0.904	20	0	0.0%
Cinnamon, bark	conv.	Vanadium	21	2.788	1.266	10	0	0.0%
Cinnamon, bark	orga.	Cobalt	24	0.252	n.c.	5	0	0.0%
Cinnamon, bark	orga.	Nickel	21	0.874	0.713	20	0	0.0%
Cinnamon, bark	orga.	Vanadium	24	1.599	0	10	0	0.0%
Clove, buds	conv.	Arsenic	14	< 0.7	n.c.	1.5	0	0.0%
Clove, buds	conv.	Nickel	14	1.6	1.2	20	0	0.0%
Cocoa, beans	orga.	Cobalt	10	0.851	0.812	5	0	0.0%
Cocoa, beans	orga.	Nickel	10	17	14.2	20	0	0.0%
Cocoa, beans	orga.	Vanadium	10	0.204	0	10	0	0.0%
Cocoa, husks	orga.	Cobalt	15	2.3	2.27	5	0	0.0%
Cocoa, husks	orga.	Nickel	15	24.9	24.8	20	8	53.3%
Cocoa, husks	orga.	Vanadium	15	1.405	1.122	10	0	0.0%
Cola Nut, seeds	conv.	Cobalt	13	0.109	n.c.	5	0	0.0%
Cola Nut, seeds	conv.	Nickel	13	5.6	4.7	20	0	0.0%
Cola Nut, seeds	conv.	Vanadium	13	0.182	0	10	0	0.0%
Common Beet	orga.	Cobalt	11	0.101	n.c.	5	0	0.0%
Common Beet	orga.	Nickel	11	0.692	n.c.	20	0	0.0%
Common Beet	orga.	Vanadium	11	0.306	0	10	0	0.0%
Common Carline Thistl, root	conv.	Cobalt	11	0.944	0.517	5	0	0.0%
Common Carline Thistl, root	conv.	Nickel	11	10.4	9.98	20	0	0.0%
Common Carline Thistl, root	conv.	Vanadium	11	6.037	2.162	10	0	0.0%
Common Ivy, leaves and herb	conv.	Arsenic	15	< 0.7	n.c.	1.5	0	0.0%
Common Wormwood, herb	conv.	Arsenic	10	1.41	n.c.	1.5	0	0.0%
Common Wormwood, herb	conv.	Nickel	11	6.79	2.49	20	0	0.0%

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	ICH Q3D Limit (10 g)		
						Max limit (ML)	No. of samples > ML	% Results > ML
Common Wormwood, herb	orga.	Cobalt	21	0.339	0.326	5	0	0.0%
Common Wormwood, herb	orga.	Nickel	22	4.28	2.91	20	0	0.0%
Common Wormwood, herb	orga.	Vanadium	22	1.715	0.646	10	0	0.0%
Cornflower, flowers	orga.	Cobalt	21	0.225	0.211	5	0	0.0%
Cornflower, flowers	orga.	Nickel	21	6.7	4.98	20	0	0.0%
Cornflower, flowers	orga.	Vanadium	21	0.708	0.543	10	0	0.0%
Couch-Grass, rhizome	conv.	Cobalt	16	0.732	0.491	5	0	0.0%
Couch-Grass, rhizome	conv.	Nickel	19	5.23	3.41	20	0	0.0%
Couch-Grass, rhizome	conv.	Vanadium	16	3.247	1.337	10	0	0.0%
Cowslip, Oxslip, flowers	orga.	Cobalt	21	0.587	n.c.	5	0	0.0%
Cowslip, Oxslip, flowers	orga.	Nickel	21	5.84	3.9	20	0	0.0%
Cowslip, Oxslip, flowers	orga.	Vanadium	21	2.381	0.682	10	0	0.0%
Cowslip, Oxslip, root	conv.	Arsenic	25	6.6	2.4	1.5	11	44.0%
Creeping Thyme, herb	conv.	Cobalt	13	9.06	3.77	5	1	7.7%
Creeping Thyme, herb	conv.	Nickel	14	235	65.1	20	8	57.1%
Creeping Thyme, herb	conv.	Vanadium	13	6.754	6.376	10	0	0.0%
Curcuma, root	conv.	Cobalt	17	0.256	0.234	5	0	0.0%
Curcuma, root	conv.	Nickel	14	0.629	n.c.	20	0	0.0%
Curcuma, root	conv.	Vanadium	17	1.064	0.794	10	0	0.0%
Daisy, flowers	orga.	Cobalt	15	1.3	0.742	5	0	0.0%
Daisy, flowers	orga.	Nickel	15	17	10	20	0	0.0%
Daisy, flowers	orga.	Vanadium	15	2.43	1.033	10	0	0.0%
Dandelion, herb	orga.	Arsenic	24	3.16	2.92	1.5	9	37.5%
Dandelion, herb	orga.	Cobalt	54	2.17	1.17	5	0	0.0%
Dandelion, herb	orga.	Nickel	54	22.9	11.8	20	1	1.9%
Dandelion, herb	orga.	Vanadium	54	7.94	4.101	10	0	0.0%
Dandelion, herb, root	conv.	Arsenic	23	2.37	n.c.	1.5	3	13.0%
Dandelion, herb, root	conv.	Cobalt	18	0.736	0.67	5	0	0.0%
Dandelion, herb, root	conv.	Nickel	22	16.6	10.9	20	0	0.0%
Dandelion, herb, root	conv.	Vanadium	18	4.05	3.209	10	0	0.0%
Dandelion, herb, root	orga.	Cobalt	26	0.869	0.734	5	0	0.0%
Dandelion, herb, root	orga.	Nickel	26	5.79	4.47	20	0	0.0%
Dandelion, herb, root	orga.	Vanadium	26	4.169	2.743	10	0	0.0%
Dandelion, root	orga.	Cobalt	20	0.599	0.501	5	0	0.0%
Dandelion, root	orga.	Nickel	20	6.91	6.58	20	0	0.0%
Dandelion, root	orga.	Vanadium	20	2.776	2.155	10	0	0.0%
Dead Nettle, flowers	conv.	Cobalt	18	2.87	0.259	5	0	0.0%
Dead Nettle, flowers	conv.	Nickel	18	39.1	3.33	20	1	5.6%
Dead Nettle, flowers	conv.	Vanadium	18	7.343	0.606	10	0	0.0%
Devil's claw, root	conv.	Arsenic	35	1.54	n.c.	1.5	1	2.9%
Devil's claw, root	conv.	Cobalt	77	0.482	0.379	5	0	0.0%
Devil's claw, root	conv.	Nickel	79	7.91	2.62	20	0	0.0%
Devil's claw, root	conv.	Vanadium	77	1.399	0.986	10	0	0.0%
Dwarf montain pine, shoot	conv.	Cobalt	12	0.353	n.c.	5	0	0.0%
Dwarf montain pine, shoot	conv.	Nickel	12	10.8	10	20	0	0.0%
Dwarf montain pine, shoot	conv.	Vanadium	12	0.407	0	10	0	0.0%
Echinacea, herb	conv.	Arsenic	33	1.05	n.c.	1.5	0	0.0%

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	ICH Q3D Limit (10 g)		
						Max limit (ML)	No. of samples > ML	% Results > ML
Echinacea, root	conv.	Arsenic	11	0.78	n.c.	1.5	0	0.0%
Elder, flowers	conv.	Arsenic	21	< 0.7	n.c.	1.5	0	0.0%
Elder, flowers	orga.	Cobalt	40	0.394	0.157	5	0	0.0%
Elder, flowers	orga.	Nickel	40	4.71	1.95	20	0	0.0%
Elder, flowers	orga.	Vanadium	40	0.954	0.437	10	0	0.0%
Elder, fruit	orga.	Cobalt	21	0.222	n.c.	5	0	0.0%
Elder, fruit	orga.	Nickel	19	2.8	1.78	20	0	0.0%
Elder, fruit	orga.	Vanadium	21	0.555	0.52	10	0	0.0%
Elecampane, rhizome	conv.	Arsenic	15	0.73	n.c.	1.5	0	0.0%
Elecampane, rhizome	conv.	Nickel	20	8.46	5.4	20	0	0.0%
Eucalyptus, leaves	orga.	Cobalt	14	0.291	n.c.	5	0	0.0%
Eucalyptus, leaves	orga.	Nickel	14	3.08	2.72	20	0	0.0%
Eucalyptus, leaves	orga.	Vanadium	14	0.274	0	10	0	0.0%
Eyebright, herb	conv.	Arsenic	12	< 0.7	n.c.	1.5	0	0.0%
Eyebright, herb	conv.	Cobalt	10	0.335	n.c.	5	0	0.0%
Eyebright, herb	conv.	Nickel	12	4.96	4.51	20	0	0.0%
Eyebright, herb	conv.	Vanadium	10	0.77	0	10	0	0.0%
Fennel, seeds	conv.	Cobalt	10	< 0.1	n.c.	5	0	0.0%
Fennel, seeds	conv.	Nickel	10	3.13	2.54	20	0	0.0%
Fennel, seeds	conv.	Vanadium	10	0.102	0	10	0	0.0%
Fennel, seeds	orga.	Arsenic	14	2.71	n.c.	1.5	2	14.3%
Fennel, seeds	orga.	Cobalt	91	0.491	0.259	5	0	0.0%
Fennel, seeds	orga.	Nickel	90	6.2	3.44	20	0	0.0%
Fennel, seeds	orga.	Vanadium	90	0.846	0.539	10	0	0.0%
Fig tree, fruit	conv.	Cobalt	12	0.165	n.c.	5	0	0.0%
Fig tree, fruit	conv.	Nickel	12	1.98	1.87	20	0	0.0%
Fig tree, fruit	conv.	Vanadium	12	0.168	0	10	0	0.0%
Fig tree, fruit	orga.	Cobalt	14	0.12	n.c.	5	0	0.0%
Fig tree, fruit	orga.	Nickel	14	3.08	2.79	20	0	0.0%
Fig tree, fruit	orga.	Vanadium	14	0.086	0	10	0	0.0%
Florence Fennel, seeds	orga.	Cobalt	15	0.353	0.307	5	0	0.0%
Florence Fennel, seeds	orga.	Nickel	15	4.51	4.39	20	0	0.0%
Florence Fennel, seeds	orga.	Vanadium	15	0.299	0.245	10	0	0.0%
Garlic, bulb	conv.	Arsenic	10	< 0.7	n.c.	1.5	0	0.0%
Giant Goldenrod, herb	orga.	Cobalt	11	0.148	n.c.	5	0	0.0%
Giant Goldenrod, herb	orga.	Nickel	11	2.95	1.94	20	0	0.0%
Giant Goldenrod, herb	orga.	Vanadium	11	0.296	0	10	0	0.0%
Ginger, rhizome	conv.	Arsenic	13	< 0.7	n.c.	1.5	0	0.0%
Ginger, rhizome	conv.	Cobalt	25	0.626	0.419	5	0	0.0%
Ginger, rhizome	conv.	Nickel	35	3.33	2.5	20	0	0.0%
Ginger, rhizome	conv.	Vanadium	25	1.711	1.174	10	0	0.0%
Ginger, rhizome	orga.	Cobalt	13	0.549	0.45	5	0	0.0%
Ginger, rhizome	orga.	Nickel	13	3.17	3.03	20	0	0.0%
Ginger, rhizome	orga.	Vanadium	13	1.249	1.218	10	0	0.0%
Ginseng, root	conv.	Arsenic	13	< 0.7	n.c.	1.5	0	0.0%
Globe Artichoke, leaves	conv.	Arsenic	39	1.4	1.3	1.5	0	0.0%
Globe Artichoke, leaves	conv.	Cobalt	66	1.96	1.07	5	0	0.0%
Globe Artichoke, leaves	conv.	Nickel	67	9.96	2.77	20	0	0.0%
Globe Artichoke, leaves	conv.	Vanadium	66	6.337	3.737	10	0	0.0%

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	ICH Q3D Limit (10 g)		
						Max limit (ML)	No. of samples > ML	% Results > ML
Globe Artichoke, leaves	orga.	Cobalt	12	0.982	0.89	5	0	0.0%
Globe Artichoke, leaves	orga.	Nickel	12	7.22	7.15	20	0	0.0%
Globe Artichoke, leaves	orga.	Vanadium	12	3.513	3.23	10	0	0.0%
Golden Rod, herb	orga.	Cobalt	15	0.183	n.c.	5	0	0.0%
Golden Rod, herb	orga.	Nickel	15	2.66	2.38	20	0	0.0%
Golden Rod, herb	orga.	Vanadium	15	0.321	0	10	0	0.0%
Hawthorn, fruit	conv.	Nickel	11	14.7	3.8	20	0	0.0%
Hawthorn, fruit	orga.	Cobalt	14	0.355	0.204	5	0	0.0%
Hawthorn, fruit	orga.	Nickel	13	8.41	2.12	20	0	0.0%
Hawthorn, fruit	orga.	Vanadium	14	0.146	0	10	0	0.0%
Hawthorn, leaves and flowers	conv.	Arsenic	11	0.862	n.c.	1.5	0	0.0%
Hawthorn, leaves and flowers	conv.	Cobalt	30	0.659	0.493	5	0	0.0%
Hawthorn, leaves and flowers	conv.	Nickel	30	6.39	3.03	20	0	0.0%
Hawthorn, leaves and flowers	conv.	Vanadium	30	2.348	1.717	10	0	0.0%
Hawthorn, leaves and flowers	orga.	Cobalt	33	0.702	0.371	5	0	0.0%
Hawthorn, leaves and flowers	orga.	Nickel	33	6.95	2.66	20	0	0.0%
Hawthorn, leaves and flowers	orga.	Vanadium	33	3.21	1.088	10	0	0.0%
Heart's Ease, herb	conv.	Cobalt	11	1.34	1.08	5	0	0.0%
Heart's Ease, herb	conv.	Nickel	13	20.9	7.91	20	1	7.7%
Heart's Ease, herb	conv.	Vanadium	11	5.761	4.009	10	0	0.0%
Henna, leaves	conv.	Nickel	31	27	7.75	20	2	6.5%
Hibiscus, flowers	orga.	Cobalt	18	1.3	0.901	5	0	0.0%
Hibiscus, flowers	orga.	Nickel	18	7.27	5.45	20	0	0.0%
Hibiscus, flowers	orga.	Vanadium	18	1.249	1.058	10	0	0.0%
Honey	conv.	Arsenic	19	< 0.7	n.c.	1.5	0	0.0%
Honey	conv.	Cobalt	19	< 0.1	n.c.	5	0	0.0%
Honey	conv.	Nickel	19	< 0.2	n.c.	20	0	0.0%
Honey	conv.	Vanadium	19	0	0	10	0	0.0%
Hops	conv.	Cobalt	19	0.257	n.c.	5	0	0.0%
Hops	conv.	Nickel	21	2.43	2.09	20	0	0.0%
Hops	conv.	Vanadium	19	1.003	0.375	10	0	0.0%
Hops	orga.	Cobalt	14	0.163	n.c.	5	0	0.0%
Hops	orga.	Nickel	14	2.34	1.98	20	0	0.0%
Hops	orga.	Vanadium	14	0.521	0.439	10	0	0.0%
Horse-Chestnut, leaves	conv.	Cobalt	11	0.283	n.c.	5	0	0.0%
Horse-Chestnut, leaves	conv.	Nickel	12	7.47	4.68	20	0	0.0%
Horse-Chestnut, leaves	conv.	Vanadium	11	0.8	0.664	10	0	0.0%
Horsetail, herb	orga.	Cobalt	54	0.894	0.71	5	0	0.0%
Horsetail, herb	orga.	Nickel	54	17.5	5.22	20	0	0.0%
Horsetail, herb	orga.	Vanadium	54	3.599	1.824	10	0	0.0%
Iceland moss, herb	conv.	Nickel	10	1.9	1.63	20	0	0.0%
Java Tea, leaves	conv.	Cobalt	12	1.2	1.16	5	0	0.0%
Java Tea, leaves	conv.	Nickel	14	0.743	0.683	20	0	0.0%
Java Tea, leaves	conv.	Vanadium	12	6.005	5.858	10	0	0.0%
Juniper, fruit	orga.	Cobalt	23	0.142	n.c.	5	0	0.0%

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	ICH Q3D Limit (10 g)		
						Max limit (ML)	No. of samples > ML	% Results > ML
Juniper, fruit	orga.	Nickel	22	4.59	3.94	20	0	0.0%
Juniper, fruit	orga.	Vanadium	23	0.106	0	10	0	0.0%
Knotgrass, herb	conv.	Cobalt	17	1.15	0.764	5	0	0.0%
Knotgrass, herb	conv.	Nickel	17	19.3	8.24	20	0	0.0%
Knotgrass, herb	conv.	Vanadium	17	3.943	2.82	10	0	0.0%
Ladies Mantle, herb	conv.	Cobalt	15	0.391	0.285	5	0	0.0%
Ladies Mantle, herb	conv.	Nickel	15	2.72	2.17	20	0	0.0%
Ladies Mantle, herb	conv.	Vanadium	15	0.549	0.394	10	0	0.0%
Ladies Mantle, herb	orga.	Cobalt	33	0.814	0.465	5	0	0.0%
Ladies Mantle, herb	orga.	Nickel	32	2.81	2.2	20	0	0.0%
Ladies Mantle, herb	orga.	Vanadium	33	4.241	0.8	10	0	0.0%
Lavender, flowers	orga.	Cobalt	41	0.612	0.397	5	0	0.0%
Lavender, flowers	orga.	Nickel	41	13.2	4.5	20	0	0.0%
Lavender, flowers	orga.	Vanadium	41	2.052	1.534	10	0	0.0%
Lemon Balm, leaves	conv.	Arsenic	27	2	n.c.	1.5	1	3.7%
Lemon Balm, leaves	orga.	Arsenic	15	2.87	n.c.	1.5	2	13.3%
Lemon Balm, leaves	orga.	Cobalt	59	1.27	0.614	5	0	0.0%
Lemon Balm, leaves	orga.	Nickel	57	14.6	4.42	20	0	0.0%
Lemon Balm, leaves	orga.	Vanadium	58	4.211	2.326	10	0	0.0%
Lemon Verbena, herb	orga.	Cobalt	19	0.484	n.c.	5	0	0.0%
Lemon Verbena, herb	orga.	Nickel	20	1.89	1.18	20	0	0.0%
Lemon Verbena, herb	orga.	Vanadium	20	2.544	0.978	10	0	0.0%
Lemongrass, leaves	orga.	Cobalt	25	0.488	0.358	5	0	0.0%
Lemongrass, leaves	orga.	Nickel	25	3.75	1.97	20	0	0.0%
Lemongrass, leaves	orga.	Vanadium	25	1.116	0.899	10	0	0.0%
Lime, flowers	conv.	Arsenic	24	< 0.7	n.c.	1.5	0	0.0%
Lime, flowers	orga.	Arsenic	11	1.84	n.c.	1.5	1	9.1%
Lime, flowers	orga.	Cobalt	50	0.235	n.c.	5	0	0.0%
Lime, flowers	orga.	Nickel	49	4.8	3.18	20	0	0.0%
Lime, flowers	orga.	Vanadium	50	0.596	0.361	10	0	0.0%
Lime, leaves	orga.	Cobalt	36	0.445	0.226	5	0	0.0%
Lime, leaves	orga.	Nickel	36	7.71	4.35	20	0	0.0%
Lime, leaves	orga.	Vanadium	36	1.238	0.645	10	0	0.0%
Liquorice, root	conv.	Cobalt	12	0.229	n.c.	5	0	0.0%
Liquorice, root	conv.	Nickel	13	1.8	1.61	20	0	0.0%
Liquorice, root	conv.	Vanadium	12	1.145	0	10	0	0.0%
Liquorice, root	orga.	Arsenic	12	2.41	n.c.	1.5	4	33.3%
Liquorice, root	orga.	Cobalt	32	0.943	0.334	5	0	0.0%
Liquorice, root	orga.	Nickel	32	16.1	2.49	20	0	0.0%
Liquorice, root	orga.	Vanadium	32	5.265	1.337	10	0	0.0%
Mallow, flowers	conv.	Cobalt	16	1.19	0.389	5	0	0.0%
Mallow, flowers	conv.	Nickel	17	9.36	7.11	20	0	0.0%
Mallow, flowers	conv.	Vanadium	16	2.05	1.134	10	0	0.0%
Mallow, flowers	orga.	Cobalt	17	1.88	0.988	5	0	0.0%
Mallow, flowers	orga.	Nickel	17	22.7	9.95	20	1	5.9%
Mallow, flowers	orga.	Vanadium	17	2.391	1.219	10	0	0.0%
Marigold, flowers	orga.	Cobalt	42	1.58	0.866	5	0	0.0%
Marigold, flowers	orga.	Nickel	41	15.7	7.15	20	0	0.0%
Marigold, flowers	orga.	Vanadium	42	5.842	3.38	10	0	0.0%

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	ICH Q3D Limit (10 g)		
						Max limit (ML)	No. of samples > ML	% Results > ML
Marsh Trefoil, leaves	conv.	Nickel	11	1.68	n.c.	20	0	0.0%
Marshmallow, root	conv.	Arsenic	20	< 0.7	n.c.	1.5	0	0.0%
Marshmallow, root	conv.	Nickel	10	3.5	2.99	20	0	0.0%
Maté, leaves	conv.	Arsenic	49	< 0.7	n.c.	1.5	0	0.0%
Maté, leaves	orga.	Cobalt	20	0.489	0.395	5	0	0.0%
Maté, leaves	orga.	Nickel	20	7.28	5.4	20	0	0.0%
Maté, leaves	orga.	Vanadium	20	0.641	0	10	0	0.0%
Mentha arvensis leaf oil	conv.	Vanadium	11	0	0	10	0	0.0%
Milk Thistle, fruit	conv.	Cobalt	26	0.514	n.c.	5	0	0.0%
Milk Thistle, fruit	conv.	Nickel	26	2.86	1.83	20	0	0.0%
Milk Thistle, fruit	conv.	Vanadium	26	2.544	0.658	10	0	0.0%
Mistletoe, herb	conv.	Cobalt	15	1.34	1.28	5	0	0.0%
Mistletoe, herb	conv.	Nickel	17	6.66	3.66	20	0	0.0%
Mistletoe, herb	conv.	Vanadium	15	0.198	0.187	10	0	0.0%
Mistletoe, herb	orga.	Cobalt	42	1.47	0.909	5	0	0.0%
Mistletoe, herb	orga.	Nickel	42	23.8	7.34	20	1	2.4%
Mistletoe, herb	orga.	Vanadium	42	0.266	0.238	10	0	0.0%
Nana mint, leaves	orga.	Cobalt	40	1.48	0.845	5	0	0.0%
Nana mint, leaves	orga.	Nickel	40	5.71	3.12	20	0	0.0%
Nana mint, leaves	orga.	Vanadium	40	5.911	3.174	10	0	0.0%
Oats, straw, green	conv.	Arsenic	13	1.11	n.c.	1.5	0	0.0%
Oats, straw, green	orga.	Cobalt	11	0.258	n.c.	5	0	0.0%
Oats, straw, green	orga.	Nickel	10	1.62	1.48	20	0	0.0%
Oats, straw, green	orga.	Vanadium	11	0.293	0	10	0	0.0%
Olive, leaves	conv.	Arsenic	28	0.97	n.c.	1.5	0	0.0%
Paeony, flowers	conv.	Cobalt	12	0.296	n.c.	5	0	0.0%
Paeony, flowers	conv.	Nickel	12	4.43	2.36	20	0	0.0%
Paeony, flowers	conv.	Vanadium	12	0.527	0.458	10	0	0.0%
Parsley, root	conv.	Nickel	10	3.92	2.35	20	0	0.0%
Passion Flower, herb	conv.	Cobalt	28	0.145	n.c.	5	0	0.0%
Passion Flower, herb	conv.	Nickel	30	10.3	4.48	20	0	0.0%
Passion Flower, herb	conv.	Vanadium	28	0.33	0.262	10	0	0.0%
Passion Flower, herb	orga.	Cobalt	17	0.401	0.377	5	0	0.0%
Passion Flower, herb	orga.	Nickel	17	4.04	3.28	20	0	0.0%
Passion Flower, herb	orga.	Vanadium	17	1.644	1.375	10	0	0.0%
Peppermint, leaves	conv.	Arsenic	18	1.13	n.c.	1.5	0	0.0%
Peppermint, leaves	conv.	Cobalt	17	0.556	0.461	5	0	0.0%
Peppermint, leaves	conv.	Nickel	17	9.46	3.64	20	0	0.0%
Peppermint, leaves	conv.	Vanadium	20	1.929	1.398	10	0	0.0%
Peppermint, leaves	orga.	Cobalt	63	1.21	0.851	5	0	0.0%
Peppermint, leaves	orga.	Nickel	63	5.13	3.92	20	0	0.0%
Peppermint, leaves	orga.	Vanadium	63	5.074	3.524	10	0	0.0%
Pu Erh Tea, leaves	conv.	Arsenic	35	1.5	n.c.	1.5	0	0.0%
Pu Erh Tea, leaves	conv.	Cobalt	13	0.431	0.358	5	0	0.0%
Pu Erh Tea, leaves	conv.	Nickel	15	9.43	9.43	20	0	0.0%
Pu Erh Tea, leaves	conv.	Vanadium	13	1.093	0.867	10	0	0.0%
Pumpkin, seeds	conv.	Arsenic	62	< 0.7	n.c.	1.5	0	0.0%
Raspberry, leaves	orga.	Cobalt	41	0.423	0.248	5	0	0.0%
Raspberry, leaves	orga.	Nickel	41	9.67	6.78	20	0	0.0%

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	ICH Q3D Limit (10 g)		
						Max limit (ML)	No. of samples > ML	% Results > ML
Raspberry, leaves	orga.	Vanadium	41	2.346	0.539	10	0	0.0%
Red vine leaves, leaves	conv.	Arsenic	27	< 0.7	n.c.	1.5	0	0.0%
Ribwort, herb	conv.	Arsenic	16	0.949	n.c.	1.5	0	0.0%
Ribwort, herb	orga.	Cobalt	34	2.75	0.575	5	0	0.0%
Ribwort, herb	orga.	Nickel	34	30.2	5.24	20	1	2.9%
Ribwort, herb	orga.	Vanadium	34	5.827	1.874	10	0	0.0%
Rock rose, herb	orga.	Cobalt	23	3.2	2.33	5	0	0.0%
Rock rose, herb	orga.	Nickel	23	5.45	5.22	20	0	0.0%
Rock rose, herb	orga.	Vanadium	23	1.241	1.148	10	0	0.0%
Roman Camomile, flowers	conv.	Cobalt	11	0.217	0.197	5	0	0.0%
Roman Camomile, flowers	conv.	Nickel	11	4.32	3.45	20	0	0.0%
Roman Camomile, flowers	conv.	Vanadium	11	0.757	0.489	10	0	0.0%
Rooibos, leaves	orga.	Cobalt	25	0.292	0.282	5	0	0.0%
Rooibos, leaves	orga.	Nickel	24	1.63	1.29	20	0	0.0%
Rooibos, leaves	orga.	Vanadium	25	0.218	0.164	10	0	0.0%
Rose Hip, shells	orga.	Cobalt	40	0.304	n.c.	5	0	0.0%
Rose Hip, shells	orga.	Nickel	37	2.4	1.25	20	0	0.0%
Rose Hip, shells	orga.	Vanadium	40	1.027	0	10	0	0.0%
Rose, petals	orga.	Cobalt	16	0.196	n.c.	5	0	0.0%
Rose, petals	orga.	Nickel	16	4.08	2	20	0	0.0%
Rose, petals	orga.	Vanadium	16	0.595	0.417	10	0	0.0%
Rosemary leaves	orga.	Cobalt	18	0.308	n.c.	5	0	0.0%
Rosemary leaves	orga.	Nickel	18	2.4	1.74	20	0	0.0%
Rosemary leaves	orga.	Vanadium	18	1.2	0.897	10	0	0.0%
Sage, leaves	conv.	Arsenic	18	1.38	n.c.	1.5	0	0.0%
Sage, leaves	conv.	Nickel	11	10	5.81	20	0	0.0%
Sage, leaves	orga.	Arsenic	11	2.06	n.c.	1.5	2	18.2%
Sage, leaves	orga.	Cobalt	50	1.57	0.578	5	0	0.0%
Sage, leaves	orga.	Nickel	50	9.24	3.84	20	0	0.0%
Sage, leaves	orga.	Vanadium	50	5.877	1.741	10	0	0.0%
Sea Buckthorn, fruit	orga.	Cobalt	15	< 0.1	n.c.	5	0	0.0%
Sea Buckthorn, fruit	orga.	Nickel	15	2.04	1.96	20	0	0.0%
Sea Buckthorn, fruit	orga.	Vanadium	15	0.136	0	10	0	0.0%
Seaweed	conv.	Arsenic	18	50.1	47.7	1.5	18	100.0%
Senna, leaves (*C. angustifolia*)	conv.	Cobalt	24	0.296	0.223	5	0	0.0%
Senna, leaves (*C. angustifolia*)	conv.	Nickel	23	1.46	1.34	20	0	0.0%
Senna, leaves (*C. angustifolia*)	conv.	Vanadium	24	1.512	0.89	10	0	0.0%
Senna, Pods (*C. angustifolia*)	conv.	Nickel	11	2.88	2.34	20	0	0.0%
Siberian ginseng, root	conv.	Cobalt	11	0.353	n.c.	5	0	0.0%
Siberian ginseng, root	conv.	Nickel	12	10.1	1.42	20	0	0.0%
Siberian ginseng, root	conv.	Vanadium	11	0.853	0.652	10	0	0.0%
Spinach, leaves	conv.	Cobalt	30	0.472	0.337	5	0	0.0%
Spinach, leaves	conv.	Nickel	30	2.65	2	20	0	0.0%
Spinach, leaves	conv.	Vanadium	30	2.132	1.303	10	0	0.0%
Spinach, leaves	orga.	Cobalt	12	0.523	0.516	5	0	0.0%
Spinach, leaves	orga.	Nickel	12	5.17	2.32	20	0	0.0%
Spinach, leaves	orga.	Vanadium	12	1.958	1.782	10	0	0.0%

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	ICH Q3D Limit (10 g)		
						Max limit (ML)	No. of samples > ML	% Results > ML
St John's wort, herb	conv.	Arsenic	14	0.725	n.c.	1.5	0	0.0%
St John's wort, herb	conv.	Cobalt	57	1.8	0.737	5	0	0.0%
St John's wort, herb	conv.	Nickel	57	12.3	3.32	20	0	0.0%
St John's wort, herb	conv.	Vanadium	57	9.331	1.85	10	0	0.0%
St John's wort, herb	orga.	Cobalt	35	0.963	0.567	5	0	0.0%
St John's wort, herb	orga.	Nickel	35	6.97	5.28	20	0	0.0%
St John's wort, herb	orga.	Vanadium	35	3.035	0.888	10	0	0.0%
Stinging Nettle	conv.	Cobalt	24	0.911	0.456	5	0	0.0%
Stinging Nettle	conv.	Nickel	24	9.52	4.66	20	0	0.0%
Stinging Nettle	conv.	Vanadium	24	4.393	1.859	10	0	0.0%
Stinging Nettle	orga.	Arsenic	26	3.33	2.96	1.5	10	38.5%
Stinging Nettle	orga.	Cobalt	121	1.15	0.374	5	0	0.0%
Stinging Nettle	orga.	Nickel	120	11.2	3.24	20	0	0.0%
Stinging Nettle	orga.	Vanadium	121	2.837	0.909	10	0	0.0%
Stinging Nettle, Dwarf Nettle, root	conv.	Nickel	11	9.29	6.6	20	0	0.0%
Strawberry, leaves	orga.	Cobalt	14	0.5	n.c.	5	0	0.0%
Strawberry, leaves	orga.	Nickel	14	6.45	3.84	20	0	0.0%
Strawberry, leaves	orga.	Vanadium	14	1.292	0.924	10	0	0.0%
Sunflower, flowers	orga.	Cobalt	12	0.277	0.239	5	0	0.0%
Sunflower, flowers	orga.	Nickel	12	11.3	7.72	20	0	0.0%
Sunflower, flowers	orga.	Vanadium	12	1.025	0.577	10	0	0.0%
Sweet Orange, peel	orga.	Cobalt	24	< 0.1	n.c.	5	0	0.0%
Sweet Orange, peel	orga.	Nickel	22	1.05	0.662	20	0	0.0%
Sweet Orange, peel	orga.	Vanadium	24	0.187	0	10	0	0.0%
Tea (Camellia sinensis)	conv.	Arsenic	130	1.98	n.c.	1.5	3	2.3%
Tea (Camellia sinensis)	conv.	Nickel	13	8.25	7.11	20	0	0.0%
Tea (Camellia sinensis)	orga.	Arsenic	11	1.24	n.c.	1.5	0	0.0%
Tea (Camellia sinensis)	orga.	Cobalt	99	2.92	0.55	5	0	0.0%
Tea (Camellia sinensis)	orga.	Nickel	99	9.7	7.1	20	0	0.0%
Tea (Camellia sinensis)	orga.	Vanadium	99	0.578	0.317	10	0	0.0%
Thyme, herb	conv.	Arsenic	27	1.02	n.c.	1.5	0	0.0%
Thyme, herb	conv.	Nickel	10	6.32	4.93	20	0	0.0%
Thyme, herb	orga.	Arsenic	16	2.94	2.1	1.5	5	31.3%
Thyme, herb	orga.	Cobalt	64	4.29	1.17	5	0	0.0%
Thyme, herb	orga.	Nickel	64	16.8	9.61	20	0	0.0%
Thyme, herb	orga.	Vanadium	64	7.988	5.271	10	0	0.0%
Turmeric, rhizome	conv.	Cobalt	10	0.507	n.c.	5	0	0.0%
Turmeric, rhizome	conv.	Nickel	12	2.11	1.59	20	0	0.0%
Turmeric, rhizome	conv.	Vanadium	10	1.374	0	10	0	0.0%
Valerian, root	conv.	Arsenic	61	1	n.c.	1.5	0	0.0%
Valerian, root	conv.	Cobalt	10	2.27	1.06	5	0	0.0%
Valerian, root	conv.	Nickel	12	7.45	6.24	20	0	0.0%
Valerian, root	conv.	Vanadium	10	6.034	0	10	0	0.0%
Valerian, root	orga.	Cobalt	19	1.02	0.363	5	0	0.0%
Valerian, root	orga.	Nickel	19	14.4	8.88	20	0	0.0%
Valerian, root	orga.	Vanadium	19	4.369	3.951	10	0	0.0%
Vanilla	conv.	Arsenic	15	< 0.7	n.c.	1.5	0	0.0%
Walnut, leaves	orga.	Cobalt	11	0.729	n.c.	5	0	0.0%
Walnut, leaves	orga.	Nickel	11	16.4	4.61	20	0	0.0%

Herbal drug	Cultivation	Element	*n*	Max (ppm)	90th percentile (ppm)	ICH Q3D Limit (10 g)		
						Max limit (ML)	No. of samples > ML	% Results > ML
Walnut, leaves	orga.	Vanadium	11	1.059	0.889	10	0	0.0%
Watercress, herb	conv.	Cobalt	10	2.13	1.44	5	0	0.0%
Watercress, herb	conv.	Nickel	10	9.33	6.98	20	0	0.0%
Watercress, herb	conv.	Vanadium	10	9.283	0	10	0	0.0%
White Mustard, seeds	conv.	Cobalt	10	0.147	n.c.	5	0	0.0%
White Mustard, seeds	conv.	Nickel	10	2.17	1.56	20	0	0.0%
White Mustard, seeds	conv.	Vanadium	10	0.181	0	10	0	0.0%
Willow herb, herb	orga.	Cobalt	10	0.156	n.c.	5	0	0.0%
Willow herb, herb	orga.	Nickel	10	1.58	1.39	20	0	0.0%
Willow herb, herb	orga.	Vanadium	10	0.5	0	10	0	0.0%
Willow, bark	conv.	Arsenic	22	< 0.7	n.c.	1.5	0	0.0%
Yarrow, flowers	orga.	Cobalt	18	0.871	0.415	5	0	0.0%
Yarrow, flowers	orga.	Nickel	18	7.97	5.99	20	0	0.0%
Yarrow, flowers	orga.	Vanadium	18	2.333	0.839	10	0	0.0%
Yarrow, herb	conv.	Cobalt	12	0.307	0.257	5	0	0.0%
Yarrow, herb	conv.	Nickel	15	9.21	4.32	20	0	0.0%
Yarrow, herb	conv.	Vanadium	12	0.508	0.479	10	0	0.0%
Yarrow, herb	orga.	Arsenic	10	0.892	n.c.	1.5	0	0.0%
Yarrow, herb	orga.	Cobalt	59	0.484	0.235	5	0	0.0%
Yarrow, herb	orga.	Nickel	60	7.2	3.69	20	0	0.0%
Yarrow, herb	orga.	Vanadium	60	1.832	0.544	10	0	0.0%
Yellow Gentian, root	conv.	Arsenic	21	< 0.7	n.c.	1.5	0	0.0%
Yellow Gentian, root	conv.	Nickel	28	24	20.8	20	5	17.9%

Appendix 3. *Findings from 2002-2007 compared to 2008-2015 for selected herbal drugs*

The arrows indicate decreases or increases of the 90th percentiles. The results for herbal drugs showing 90th percentiles above the Ph. Eur. limit are shown in bold.

Cadmium

		2008-2015			2002-2007				
Product	**Cultivation**	**No. of samples analysed**	**Max value (ppm)**	**90th percentile (ppm)**	**No. of samples analysed**	**Max value (ppm)**	**90th percentile (ppm)**	**Difference > 10 %**	**Difference > 20 %**
Angelica, root	conv.	112	0.90	0.73	20	1.16	0.76	→	→
Aniseed	orga.	45	0.15	0.13	41	0.14	0.12	↑	→
Arnica, flowers	conv.	120	1.25	0.49	69	1.69	0.82	↓	↓
Birch, leaves	conv.	175	1.17	0.61	69	0.93	0.66	→	→
Birch, leaves	orga.	68	1.04	0.61	19	0.81	0.61	→	→
Black cohosh, root	conv.	22	0.69	0.21	19	0.27	0.21	→	→
Bladderwrack	**conv.**	33	1.00	0.83	25	1.45	1.11	↓	↓
Burdock, root	conv.	26	0.38	0.31	32	0.42	0.36	↓	→
Camomile, flowers	conv.	283	2.87	0.61	75	0.76	0.56	→	→
Camomile, flowers	orga.	178	1.21	0.58	34	0.52	0.48	↑	→
Cardamom, fruit	orga.	15	0.35	0.28	17	0.34	0.27	→	→
Chondria	conv.	16	0.60	0.36	14	0.62	0.40	→	→
Cinnamon, bark	conv.	55	0.59	0.53	48	0.64	0.40	↑	↑
Cinnamon, bark	orga.	68	0.36	0.33	33	0.27	0.16	↑	↑
Common Ivy, leaves and herb	conv.	81	0.81	0.51	25	0.50	0.39	↑	↑
Common Wormwood, herb	conv.	60	0.84	0.63	46	1.10	0.93	↓	↓
Couch-Grass, rhizome	conv.	37	0.48	0.25	30	0.26	0.18	↑	↑
Cowslip, Oxslip, root	conv.	85	0.63	0.25	18	0.27	0.19	↑	↑
Curcuma, root	conv.	35	0.66	0.28	19	0.35	0.19	↑	↑
Dandelion, herb	orga.	80	0.69	0.43	38	1.04	0.58	↓	↓
Dandelion, herb, root	conv.	89	0.95	0.52	14	0.51	0.35	↑	↑
Dandelion, root	orga.	63	0.57	0.37	17	0.38	0.28	↑	↑
Devil's claw, root	conv.	155	1.28	0.85	96	2.73	0.68	↑	↑
Devil's claw, root	conv.	154	0.13	0.12	95	0.21	0.12	→	→
Echinacea, root	conv.	47	0.94	0.47	79	2.54	0.51	→	→
Eyebright, herb	**conv.**	66	1.58	0.97	**31**	**1.40**	**1.14**	↓	→
Fumitory, herb	**conv.**	107	2.91	1.36	**15**	**1.78**	**1.71**	↓	↓
Giant Goldenrod, herb	conv.	29	0.54	0.39	20	0.57	0.41	→	→
Ginger, rhizome	conv.	92	0.65	0.41	92	0.64	0.39	→	→
Ginseng, root	conv.	77	0.26	0.17	81	0.35	0.19	↓	→
Globe Artichoke, leaves	conv.	130	0.58	0.40	168	0.74	0.43	→	→
Globe Artichoke, leaves	orga.	11	0.31	0.28	42	0.70	0.24	↑	→
Golden Rod, herb	conv.	105	1.03	0.85	55	1.05	0.91	→	→
Golden Rod, herb	orga.	18	1.15	0.79	18	0.76	0.68	↑	→
Hawthorn, leaves and flowers	conv.	143	0.51	0.18	98	0.35	0.22	↓	→
Heart's Ease, herb	**conv.**	**55**	**2.15**	**1.77**	**16**	**2.00**	**1.30**	↑	↑
Hibiscus, flowers	conv.	178	0.37	0.15	32	0.37	0.17	→	→
Horsetail, herb	conv.	83	0.32	0.15	49	0.63	0.40	↓	↓
Horsetail, herb	orga.	60	1.44	0.18	21	0.22	0.20	→	→
Iceland moss, herb	conv.	105	1.57	0.49	34	0.61	0.44	↑	→

		2008-2015			2002-2007				
Product	**Cultivation**	**No. of samples analysed**	**Max value (ppm)**	**90th percentile (ppm)**	**No. of samples analysed**	**Max value (ppm)**	**90th percentile (ppm)**	**Difference > 10 %**	**Difference > 20 %**
Linseed	conv.	10	0.41	0.40	28	0.50	0.42	→	→
Lovage, root	conv.	57	0.56	0.38	29	0.61	0.52	↓	↓
Mallow, flowers	conv.	40	0.62	0.35	12	0.48	0.44	↓	↓
Mallow, flowers	orga.	24	0.31	0.29	14	0.92	0.41	↓	↓
Mallow, leaves	conv.	81	0.55	0.34	103	3.61	0.40	↓	→
Marigold, flowers	conv.	71	0.22	0.19	95	1.09	0.52	↓	↓
Marshmallow, leaves	conv.	21	0.73	0.50	12	1.31	0.81	↓	↓
Marshmallow, root	conv.	104	0.85	0.71	27	0.95	0.62	↑	→
Maté, leaves	conv.	75	1.12	0.57	18	0.64	0.41	↑	↑
Meadowsweet, flowers	conv.	18	0.81	0.67	13	0.47	0.47	↑	↑
Meadowsweet, herb	conv.	41	1.19	0.76	15	1.08	0.36	↑	↑
Milk Thistle, fruit	conv.	56	0.80	0.44	33	0.51	0.37	↑	→
Mistletoe, herb	conv.	171	1.39	1.01	223	1.81	0.87	↑	→
Mistletoe, herb	orga.	47	0.56	0.40	25	3.16	0.64	↓	↓
Oak, bark	conv.	52	0.52	0.45	14	0.31	0.28	↑	↑
Passion Flower, herb	conv.	107	0.40	0.26	44	0.66	0.45	↓	↓
Peppermint, leaves	conv.	75	0.55	0.21	52	0.42	0.20	→	→
Raspberry, leaves	conv.	21	0.42	0.28	37	0.87	0.42	↓	↓
Raspberry, leaves	orga.	75	0.57	0.35	28	0.49	0.40	↓	→
Rest Harrow, root	conv.	38	0.54	0.17	31	0.15	0.13	↑	↑
Ribwort, herb	conv.	264	1.05	0.40	55	0.50	0.37	→	→
Ribwort, herb	orga.	41	1.00	0.65	18	0.26	0.18	↑	↑
Seaweed	**conv.**	18	**7.73**	**5.71**	**21**	**6.60**	**5.71**	→	→
Sheep's bane, herb	**conv.**	52	1.40	0.68	**11**	**1.09**	**1.06**	↓	↓
Silver Lime, flowers	conv.	160	0.43	0.14	42	0.21	0.15	→	→
Spinach, leaves	**conv.**	**30**	**1.75**	**1.53**	**53**	**3.25**	**1.57**	→	→
St John's wort, herb	conv.	330	3.18	0.93	163	1.24	0.96	→	→
St John's wort, herb	orga.	36	1.36	0.81	25	2.51	0.66	↑	↑
Stinging Nettle, Dwarf Nettle, root	conv.	48	0.22	0.15	34	0.20	0.13	↑	→
Thyme, herb	conv.	305	1.84	0.53	57	0.70	0.61	↓	→
Thyme, herb	orga.	111	0.68	0.29	35	0.43	0.20	↑	↑
Valerian, root	conv.	382	0.39	0.21	119	0.54	0.28	↓	↓
Watercress, herb	**conv.**	**65**	**1.72**	**1.08**	**31**	**6.53**	**1.27**	↓	→
Willow, bark	**conv.**	**155**	**8.21**	**2.74**	61	3.53	1.70	↑	↑
Yarrow, herb	conv.	52	1.17	0.93	23	0.98	0.60	↑	↑
Yarrow, herb	orga.	72	0.66	0.45	29	0.63	0.44	→	→
Yellow Gentian, root	conv.	63	0.56	0.37	110	0.98	0.35	→	→
Mean value (90th percentile)		**6729**		**0.60**	**3504**		**0.59**	→	→

Lead

		2008-2015			2002-2007				
Product	**Cultivation**	**No. of samples analysed**	**Max value (ppm)**	**90th percentile (ppm)**	**No. of samples analysed**	**Max value (ppm)**	**90th percentile (ppm)**	**Difference > 10 %**	**Difference > 20 %**
Alder Buckthorn, bark	conv.	76	3.86	2.06	22	4.42	2.03	→	→
Angelica, root	conv.	97	3.58	2.15	16	2.37	1.79	↑	↑
Arnica, flowers	conv.	117	23.60	2.76	63	4.04	2.17	↑	↑
Birch, leaves	conv.	177	34.20	3.02	64	3.93	2.47	↑	↑
Birch, leaves	orga.	68	7.03	0.99	19	2.33	1.57	↓	↓
Black cohosh, root	conv.	22	4.58	3.44	19	1.56	1.46	↑	↑
Bladderwrack	conv.	33	1.58	1.13	25	3.58	1.32	↓	→
Blond Psyllium, husk	conv.	252	15.00	1.91	68	2.30	1.85	→	→
Butcher's Broom, rhizome	conv.	37	8.50	3.67	12	6.18	2.86	↑	↑
Camomile, flowers	conv.	270	8.60	1.54	62	3.12	1.80	↓	→
Cinnamon, bark	**conv.**	61	8.13	3.60	**49**	**11.89**	**5.59**	↓	↓
Common Ivy, leaves and herb	conv.	81	5.64	1.36	24	1.91	1.48	→	→
Common Wormwood, herb	conv.	53	3.28	1.31	25	1.82	1.69	↓	↓
Couch-Grass, rhizome	conv.	36	2.51	1.75	31	1.42	1.04	↑	↑
Cowslip, Oxslip, root	conv.	91	7.52	5.00	17	4.40	3.05	↑	↑
Creeping Thyme, herb	conv.	33	3.66	1.86	12	10.21	4.16	↓	↓
Dandelion, herb	orga.	80	5.85	2.71	38	3.96	2.57	→	→
Dandelion, root	orga.	62	4.17	1.93	17	3.28	1.06	↑	↑
Devil's claw, root	conv.	155	1.28	0.85	96	2.73	0.68	↑	↑
Echinacea, root	conv.	38	4.12	2.50	81	3.44	1.89	↑	↑
Elder, flowers	conv.	57	9.20	3.09	32	3.25	2.90	→	→
Eyebright, herb	conv.	58	3.39	1.50	39	47.37	2.70	↓	↓
Ginger, rhizome	orga.	87	1.67	0.92	15	4.12	1.71	↓	↓
Ginger, rhizome	conv.	75	2.60	1.16	86	2.58	1.53	↓	↓
Ginkgo, leaves	conv.	64	8.11	4.64	17	4.95	4.81	→	→
Globe Artichoke, leaves	conv.	129	2.82	1.80	167	32.16	2.54	↓	↓
Golden Rod, herb	conv.	80	2.50	2.34	53	2.50	1.09	↑	↑
Hawthorn, leaves and flowers	conv.	177	34.30	3.54	181	98.30	4.71	↓	↓
Heart's Ease, herb	conv.	32	3.28	2.40	14	1.12	1.00	↑	↑
Hops	conv.	193	3.97	1.63	74	3.53	1.06	↑	↑
Horsetail, herb	conv.	84	9.24	1.04	47	21.45	0.94	↑	→
Iceland moss, herb	**conv.**	112	13.40	7.28	**35**	**13.87**	**11.06**	↓	↓
Java Tea, leaves	conv.	56	3.66	2.44	24	4.02	3.67	↓	↓
Ladies Mantle, herb	conv.	22	3.01	0.84	19	1.10	1.05	↓	↓
Lavender, flowers	orga.	53	6.31	1.86	18	1.48	0.85	↑	↑
Lemon Balm, leaves	conv.	125	2.51	1.40	57	10.40	2.77	↓	↓
Lemon Balm, leaves	orga.	282	5.33	2.55	27	1.53	1.26	↑	↑
Lovage, root	conv.	54	3.59	2.78	25	1.15	0.90	↑	↑
Mallow, leaves	conv.	80	11.00	3.78	103	57.42	3.55	→	→
Marigold, flowers	conv.	71	7.94	1.66	93	2.05	1.78	→	→
Marigold, flowers	orga.	66	9.23	1.61	28	1.67	0.86	↑	↑
Marshmallow, root	conv.	102	25.90	1.70	29	5.00	1.39	↑	↑
Mistletoe, herb	conv.	168	16.00	1.07	200	2.89	1.66	↓	↓
Nana mint, leaves	orga.	40	1.90	1.34	16	2.01	1.56	↓	→

		2008-2015			2002-2007				
Product	**Cultivation**	**No. of samples analysed**	**Max value (ppm)**	**90th percentile (ppm)**	**No. of samples analysed**	**Max value (ppm)**	**90th percentile (ppm)**	**Difference > 10 %**	**Difference > 20 %**
Olive, leaves	conv.	51	1.72	1.39	16	3.85	3.20	↓	↓
Passion Flower, herb	conv.	105	3.40	1.33	44	1.98	1.30	→	→
Peppermint, leaves	conv.	84	1.35	1.26	52	65.00	1.64	↓	↓
Peppermint, leaves	orga.	267	10.10	1.42	58	2.63	1.21	↑	→
Raspberry, leaves	orga.	75	16.60	1.60	28	3.46	1.73	→	→
Raspberry, leaves	conv.	18	1.90	1.34	38	2.44	1.44	→	→
Red vine leaves, leaves	conv.	169	3.30	1.22	54	3.40	2.10	↓	↓
Rest Harrow, root	conv.	36	2.29	1.82	29	3.18	1.57	↑	→
Ribwort, herb	conv.	264	6.00	1.71	53	5.24	1.22	↑	↑
Safflower, flowers	**conv.**	32	132.00	2.29	**31**	**258.90**	**53.12**	↓	↓
Sage, leaves	conv.	99	3.57	1.37	51	6.47	3.72	↓	↓
Sage, leaves	orga.	117	4.48	1.68	46	2.10	1.12	↑	↑
Senna, leaves (C. acutifolia)	conv.	179	1.54	0.95	30	0.93	0.59	↑	↑
Sheep's bane, herb	conv.	52	3.97	1.46	11	4.09	3.98	↓	↓
Silver Lime, flowers	conv.	161	24.00	3.14	42	15.15	2.99	→	→
Spinach, leaves	conv.	30	1.03	0.81	52	6.80	1.20	↓	↓
St John's wort, herb	conv.	258	17.50	4.10	156	14.51	3.03	↑	↑
Stinging Nettle, Dwarf Nettle, herb	**conv.**	**119**	**510.00**	**14.80**	72	6.81	2.14	↑	↑
Stinging Nettle, Dwarf Nettle, herb	orga.	86	7.62	3.29	57	4.28	1.13	↑	↑
Stinging Nettle, Dwarf Nettle, root	**conv.**	50	19.30	5.58	**43**	**4249.60**	**7.06**	↓	↓
Strawberry, leaves	conv.	81	2.70	1.08	28	3.91	2.76	↓	↓
Strawberry, leaves	orga.	37	6.53	3.01	28	5.05	1.38	↑	↑
Tea (Camellia sinensis)	conv.	265	6.52	2.44	27	5.66	2.40	→	→
Thyme, herb	conv.	294	3.50	1.94	41	4.73	2.18	↓	→
Thyme, herb	orga.	113	3.68	2.29	35	2.16	1.26	↑	↑
Valerian, root	conv.	389	10.70	3.52	119	8.81	2.69	↑	↑
Watercress, herb	**conv.**	**65**	**10.60**	**7.44**	**30**	**13.65**	**6.50**	↑	→
Willow, bark	**conv.**	61	4.66	1.92	**43**	**6.92**	**5.56**	↓	↓
Yellow Gentian, root	conv.	61	4.40	2.20	108	19.23	2.24	→	→
Mean value (90th Percentile)		**7724**		**2.44**	**3581**		**3.05**	↓	→

Mercury

		2008-2015			2002-2007				
Product	**Cultivation**	**No. of samples analysed**	**Max value (ppm)**	**90th percentile (ppm)**	**No. of samples analysed**	**Max value (ppm)**	**90th percentile (ppm)**	**Difference > 10 %**	**Difference > 20 %**
Ginkgo, leaves	**conv.**	**68**	**0.21**	**0.21**	12	0.09	0.06	↑	↑
Valerian, root	conv.	347	0.26	0.1	68	0.06	0.05	↑	↑
Olive, leaves	conv.	48	0.07	0.05	12	0.06	0.06	↓	→
Common Ivy, leaves and herb	conv.	79	0.09	0.04	17	0.03	0.03	↑	↑
Mallow, leaves	conv.	79	0.06	0.04	66	0.04	0.03	↑	↑
Red vine leaves, leaves	conv.	169	0.08	0.04	29	0.06	0.04	→	→
Rosemary leaves	conv.	15	0.08	0.04	13	0.06	0.05	↓	→
Thyme, herb	conv.	285	0.08	0.04	22	0.06	0.04	→	→
Iceland moss, herb	conv.	96	0.04	0.03	21	0.05	0.03	→	→
Mistletoe, herb	conv.	155	0.04	0.03	48	0.06	0.04	↓	↓
Sage, leaves	conv.	98	0.04	0.03	27	0.06	0.04	↓	↓
Lemon Balm, leaves	conv.	113	0.05	0.02	38	0.05	0.04	↓	↓
Peppermint, leaves	conv.	59	0.04	0.02	38	0.06	0.04	↓	↓
Stinging Nettle, Dwarf Nettle, herb	conv.	112	0.03	0.02	50	0.17	0.02	→	→
Mean value (90th Percentile)		**1723**		**0.05**	**461**		**0.04**	↑	↑

Appendix 4. *Results for cadmium, cobalt, lead, mercury, nickel and vanadium in essential oils*

Essential oil	Cultivation	Element	*n*	No. of positive samples (> LOQ)	Max (ppm)	90th percentile (ppm)	Ph. Eur. 9.0 Limit			ICH Q3D Limit (10 g)		
							Max limit (ML)	No. of samples > ML	% Results > ML	Max limit (ML)	No. of samples > ML	% Results > ML
Bitter-fennel oil	conv.	Cadmium	5	0	0	0	1	0	0.0%	0.5	0	0.0%
Bitter-fennel oil	conv.	Lead	5	0	0	0	5	0	0.0%	0.5	0	0.0%
Bitter-fennel oil	conv.	Mercury	5	2	0.03	0	0.1	0	0.0%	3	0	0.0%
Cajeput oil	conv.	Cadmium	3	0	0	0	1	0	0.0%	0.5	0	0.0%
Cajeput oil	conv.	Lead	3	0	0	0	5	0	0.0%	0.5	0	0.0%
Cajeput oil	conv.	Mercury	3	0	0	0	0.1	0	0.0%	3	0	0.0%
Camomile flower oil (C. recutita)	conv.	Cadmium	1	0	0	0	1	0	0.0%	0.5	0	0.0%
Camomile flower oil (C. recutita)	conv.	Lead	1	0	0	0	5	0	0.0%	0.5	0	0.0%
Camomile flower oil (C. recutita)	conv.	Mercury	1	1	0.03	0	0.1	0	0.0%	3	0	0.0%
Caraway, oil	conv.	Cadmium	18	0	0	0	1	0	0.0%	0.5	0	0.0%
Caraway, oil	conv.	Lead	18	0	0	0	5	0	0.0%	0.5	0	0.0%
Caraway, oil	conv.	Mercury	17	0	0	0	0.1	0	0.0%	3	0	0.0%
Caraway, oil	conv.	Vanadium	4	0	0	0				10	0	0.0%
Clove oil	conv.	Cadmium	5	0	0	0	1	0	0.0%	0.5	0	0.0%
Clove oil	conv.	Lead	5	0	0	0	5	0	0.0%	0.5	0	0.0%
Clove oil	conv.	Mercury	5	1	0.02	0	0.1	0	0.0%	3	0	0.0%
Eucalyptus oil	conv.	Cadmium	32	0	0	0	1	0	0.0%	0.5	0	0.0%
Eucalyptus oil	conv.	Lead	32	0	0	0	5	0	0.0%	0.5	0	0.0%
Eucalyptus oil	conv.	Mercury	32	0	0	0	0.1	0	0.0%	3	0	0.0%
Geranium oil	conv.	Cadmium	3	0	0	0	1	0	0.0%	0.5	0	0.0%
Geranium oil	conv.	Lead	3	0	0	0	5	0	0.0%	0.5	0	0.0%
Geranium oil	conv.	Mercury	3	2	0.07	0	0.1	0	0.0%	3	0	0.0%
Geranium oil	orga.	Cadmium	1	0	0	0	1	0	0.0%	0.5	0	0.0%
Geranium oil	orga.	Lead	1	0	0	0	5	0	0.0%	0.5	0	0.0%
Geranium oil	orga.	Mercury	1	0	0	0	0.1	0	0.0%	3	0	0.0%
Ginger oil	conv.	Cadmium	1	0	0	0	1	0	0.0%	0.5	0	0.0%
Ginger oil	conv.	Lead	1	0	0	0	5	0	0.0%	0.5	0	0.0%
Ginger oil	conv.	Mercury	1	0	0	0	0.1	0	0.0%	3	0	0.0%

Essential oil	Cultivation	Element	*n*	No. of positive samples (> LOQ)	Max (ppm)	90th per-centile (ppm)	Ph. Eur. 9.0 Limit			ICH Q3D Limit (10 g)		
							Max limit (ML)	No. of samples > ML	% Results > ML	Max limit (ML)	No. of samples > ML	% Results > ML
Juniper oil	conv.	Cadmium	1	0	0	0	1	0	0.0%	0.5	0	0.0%
Juniper oil	conv.	Lead	1	0	0	0	5	0	0.0%	0.5	0	0.0%
Juniper oil	conv.	Mercury	1	0	0	0	0.1	0	0.0%	3	0	0.0%
Lavender oil	conv.	Cadmium	10	0	0	0	1	0	0.0%	0.5	0	0.0%
Lavender oil	conv.	Lead	10	0	0	0	5	0	0.0%	0.5	0	0.0%
Lavender oil	conv.	Mercury	10	1	0.02	0	0.1	0	0.0%	3	0	0.0%
Lemon oil	conv.	Cadmium	8	0	0	0	1	0	0.0%	0.5	0	0.0%
Lemon oil	conv.	Cobalt	1	1	0.544	0				5	0	0.0%
Lemon oil	conv.	Lead	8	0	0	0	5	0	0.0%	0.5	0	0.0%
Lemon oil	conv.	Mercury	7	0	0	0	0.1	0	0.0%	3	0	0.0%
Lemon oil	conv.	Vanadium	4	0	0	0				10	0	0.0%
Litsea cubeba oil	conv.	Cadmium	1	0	0	0	1	0	0.0%	0.5	0	0.0%
Litsea cubeba oil	conv.	Lead	1	0	0	0	5	0	0.0%	0.5	0	0.0%
Litsea cubeba oil	conv.	Mercury	1	0	0	0	0.1	0	0.0%	3	0	0.0%
Marjoran oil	conv.	Cadmium	3	0	0	0	1	0	0.0%	0.5	0	0.0%
Marjoran oil	conv.	Lead	3	0	0	0	5	0	0.0%	0.5	0	0.0%
Marjoran oil	conv.	Mercury	3	0	0	0	0.1	0	0.0%	3	0	0.0%
Mentha arvensis leaf oil	conv.	Cadmium	10	0	0	0	1	0	0.0%	0.5	0	0.0%
Mentha arvensis leaf oil	conv.	Lead	10	0	0	0	5	0	0.0%	0.5	0	0.0%
Mentha arvensis leaf oil	conv.	Mercury	9	0	0	0	0.1	0	0.0%	3	0	0.0%
Mentha arvensis leaf oil	conv.	Vanadium	11	0	0	0				10	0	0.0%
Myrrh, oil	conv.	Cadmium	12	0	0	0	1	0	0.0%	0.5	0	0.0%
Myrrh, oil	conv.	Cobalt	2	2	0.158	0				5	0	0.0%
Myrrh, oil	conv.	Lead	13	2	0.96	0	5	0	0.0%	0.5	2	15.4%
Myrrh, oil	conv.	Mercury	13	0	0	0	0.1	0	0.0%	3	0	0.0%
Myrrh, oil	conv.	Nickel	2	2	1.64	0				20	0	0.0%
Myrrh, oil	conv.	Vanadium	2	2	0.671	0				10	0	0.0%
Peppermint oil	conv.	Cadmium	15	0	0	0	1	0	0.0%	0.5	0	0.0%
Peppermint oil	conv.	Lead	15	0	0	0	5	0	0.0%	0.5	0	0.0%

Essential oil	Cultivation	Element	*n*	No. of positive samples (> LOQ)	Max (ppm)	90th per-centile (ppm)	Ph. Eur. 9.0 Limit			ICH Q3D Limit (10 g)		
							Max limit (ML)	No. of samples > ML	% Results > ML	Max limit (ML)	No. of samples > ML	% Results > ML
Peppermint oil	conv.	Mercury	15	1	0.02	0	0.1	0	0.0%	3	0	0.0%
Pini needle oil	conv.	Cadmium	8	0	0	0	1	0	0.0%	0.5	0	0.0%
Pini needle oil	conv.	Lead	8	0	0	0	5	0	0.0%	0.5	0	0.0%
Pini needle oil	conv.	Mercury	8	0	0	0	0.1	0	0.0%	3	0	0.0%
Pinus pinaster oil	conv.	Cadmium	4	0	0	0	1	0	0.0%	0.5	0	0.0%
Pinus pinaster oil	conv.	Lead	4	1	0.62	0	5	0	0.0%	0.5	1	25.0%
Pinus pinaster oil	conv.	Mercury	4	0	0	0	0.1	0	0.0%	3	0	0.0%
Pinus pumilio oil	conv.	Cadmium	10	0	0	0	1	0	0.0%	0.5	0	0.0%
Pinus pumilio oil	conv.	Lead	10	0	0	0	5	0	0.0%	0.5	0	0.0%
Pinus pumilio oil	conv.	Mercury	11	4	0.03	0	0.1	0	0.0%	3	0	0.0%
Pinus pumilio oil	conv.	Vanadium	6	0	0	0				10	0	0.0%
Rose oil	conv.	Cadmium	7	0	0	0	1	0	0.0%	0.5	0	0.0%
Rose oil	conv.	Lead	9	2	8.65	0	5	1	11.1%	0.5	2	22.2%
Rose oil	conv.	Mercury	7	2	0.05	0	0.1	0	0.0%	3	0	0.0%
Rosemary oil	conv.	Cadmium	8	0	0	0	1	0	0.0%	0.5	0	0.0%
Rosemary oil	conv.	Lead	8	0	0	0	5	0	0.0%	0.5	0	0.0%
Rosemary oil	conv.	Mercury	8	2	0.03	0	0.1	0	0.0%	3	0	0.0%
Sage oil	conv.	Cadmium	4	0	0	0	1	0	0.0%	0.5	0	0.0%
Sage oil	conv.	Lead	4	0	0	0	5	0	0.0%	0.5	0	0.0%
Sage oil	conv.	Mercury	4	2	0.02	0	0.1	0	0.0%	3	0	0.0%
Star anise oil	conv.	Cadmium	19	0	0	0	1	0	0.0%	0.5	0	0.0%
Star anise oil	conv.	Lead	19	0	0	0	5	0	0.0%	0.5	0	0.0%
Star anise oil	conv.	Mercury	20	1	0.02	0	0.1	0	0.0%	3	0	0.0%
Star anise oil	conv.	Vanadium	2	0	0	0				10	0	0.0%
Tea Tree oil	conv.	Cadmium	9	0	0	0	1	0	0.0%	0.5	0	0.0%
Tea Tree oil	conv.	Lead	9	0	0	0	5	0	0.0%	0.5	0	0.0%
Tea Tree oil	conv.	Mercury	9	2	0.04	0	0.1	0	0.0%	3	0	0.0%
Thyme oil	conv.	Cadmium	29	0	0	0	1	0	0.0%	0.5	0	0.0%
Thyme oil	conv.	Lead	29	0	0	0	5	0	0.0%	0.5	0	0.0%

Essential oil	Cultivation	Element	*n*	No. of positive samples (> LOQ)	Max (ppm)	90th percentile (ppm)	Ph. Eur. 9.0 Limit			ICH Q3D Limit (10 g)		
							Max limit (ML)	No. of samples > ML	% Results > ML	Max limit (ML)	No. of samples > ML	% Results > ML
Thyme oil	conv.	Mercury	29	19	0.13	0.08	0.1	1	3.4%	3	0	0.0%
Valerian root oil	conv.	Cadmium	1	0	0	0	1	0	0.0%	0.5	0	0.0%
Valerian root oil	conv.	Lead	1	0	0	0	5	0	0.0%	0.5	0	0.0%
Valerian root oil	conv.	Mercury	1	0	0	0	0.1	0	0.0%	3	0	0.0%

Calibration of pertussis toxin BRP batch 1 in a standardised CHO cell-based clustering assay

K. Markey[1], A. Douglas-Bardsley[1], J. Hockley[1],
D. Le Tallec[2], A. Costanzo[2]

ABSTRACT

The European Pharmacopoeia (Ph. Eur.) pertussis toxin (PT) Biological Reference Preparation (BRP) is used as a working standard for safety testing of acellular pertussis vaccines as prescribed in the Ph. Eur. monographs 1356 "Pertussis vaccine (acellular, component, adsorbed)" and 1595 "Pertussis vaccine (acellular, co-purified, adsorbed)".

The BRP was calibrated in 2006 in the murine histamine sensitisation test (HIST) against the World Health Organization (WHO) 1st International Standard (IS) for PT. In recent years, there have been increasing efforts to replace the in vivo test with in vitro methods. The Chinese hamster ovary (CHO) cell clustering assay has been used for many years by manufacturers to monitor residual PT activity in detoxified non-adjuvanted bulks. More recently a standardised protocol has been developed for this assay and a PT reference preparation was needed. Due to low stocks, the WHO 1st International Standard for Pertussis Toxin (JNIH-5) needed to be replaced and therefore a joint study between the European Directorate for the Quality of Medicines & HealthCare (EDQM) and WHO was initiated to calibrate the PT BRP for the CHO clustering assay and to replace the IS.

The collaborative study involved 14 laboratories from Europe, North America and Asia. The outcome of the study confirmed that the BRP is suitable for use as a reference preparation in the CHO clustering assay. The material was assigned a potency of 1360 IU per vial for the CHO clustering assay.

KEYWORDS

Pertussis toxin, in vitro assay, CHO cell clustering assay, Biological Reference Preparation, BRP, biological standardisation, collaborative study, European Pharmacopoeia.

1. INTRODUCTION

Residual pertussis toxin (PT) in acellular pertussis (aP) vaccines is currently tested using the murine histamine sensitisation test (HIST), an assay that has a high intra- and inter-laboratory variability and induces high distress in the animals. This test is therefore considered as a priority for replacement by *in vitro* alternatives [1], in particular following the requirements of Directive 2010/63/EC on the protection of animals used for scientific purposes. An *in vitro* test, based on morphological changes induced by PT to Chinese hamster ovary (CHO)-K1 cells in culture, was described in 1983 by Hewlett et al [2]. This assay is very sensitive and has been used for some time by manufacturers to monitor residual PT content in detoxified non-

1 K. Markey, A. Douglas-Bardsley, J. Hockley. National Institute for Biological Standards and Control (NIBSC), Blanche Lane, South Mimms, Potters Bar, EN6 3QG, UK.

2 D. Le Tallec, A. Costanzo (corresponding author: mail to angele.costanzo@edqm.eu). European Directorate for the Quality of Medicines & HealthCare, Department of Biological Standardisation, OMCL Network & HealthCare (DBO), EDQM - Council of Europe, 7 allée Kastner, CS 30026 F-67081 Strasbourg, France.

adjuvanted bulks, as described in the European Pharmacopoeia (Ph. Eur.) monographs 1356 “Pertussis vaccine (acellular, component, adsorbed)” [3] and 1595 “Pertussis vaccine (acellular, co-purified, adsorbed)” [4].

Although the use of this assay had been attempted in the past for calibration purposes [5-7], it was generally found to have a greater variability than the *in vivo* method. Significant differences in the methods used between participating laboratories were believed to be the cause of the high variability. Therefore, a standardised protocol for the CHO clustering assay was suggested to minimise this inter-laboratory variability [1].

In a recent collaborative study (BSP114) organised by the European Directorate for the Quality of Medicines & HealthCare (EDQM) under the aegis of the Biological Standardisation Programme (BSP), a standardised method was found to show good inter-laboratory reproducibility [8]. Based on these results, the standardised method has been proposed for inclusion in the calibration of future PT reference preparations. Also, a modified method which allows adjuvanted end-products to be tested was established during the same study. Discussions have started for the inclusion of these standard methods in the appropriate Ph. Eur. texts.

The current Biological Reference Preparation (BRP) for PT (batch 1) was established in 2000 for the HIST [9] and was calibrated in International Units (IU) relative to the WHO 1st International Standard (IS) for pertussis toxin (JNIH-5) in 2009 [7]. Following the completion of the BSP114 collaborative study in 2015, the BSP Steering Committee endorsed a project – code BSP145 – to calibrate the PT BRP for *in vitro* use in the CHO clustering assay. At the same time, the stocks of JNIH-5 were running low and a replacement batch was needed. It was therefore decided to run a joint EDQM-WHO study to calibrate both the PT BRP and the WHO replacement reference preparation. The PT BRP was only included in the CHO assay, whereas the candidate 2nd IS was calibrated for the HIST and CHO assays, and also assessed in various other *in vitro* tests.

The CHO-K1 cell clustering method applied during the present study was the same as that used for the BSP114 collaborative study [8] and a detailed standard protocol was provided to participants. Briefly, cells were to be transferred to 24-well cell culture plates at a concentration low enough to ensure that the culture would remain sub-confluent throughout the assay incubation period. The reconstituted PT samples were diluted serially in CHO-K1 culture media, added to the cells, and the assay plates incubated for 48 hours in a CO_2 incubator. When the incubation time was complete, the plates were scored by microscopy by two trained operators for the presence of distinct cluster formations: a score of 10 or more clusters per well being considered positive, whereas less than 10 clusters per well was assigned a negative score.

The experimental phase ran from April 2016 to January 2017. Detailed results for the candidate 2nd IS are described in a separate document [10]. Following the study, it was established as the WHO 2nd IS for pertussis toxin and will thus be referred to as 2nd IS hereunder. The present report focuses on the results obtained for the PT BRP.

JNIH-5 being the 1st IS for PT, it had originally been attributed a unique arbitrary unitage, i.e. 10 000 IU per ampoule for both the HIST and the CHO clustering assay. In the present study, the BRP, the 2nd IS and JNIH-5 were included in all assays in order to calculate a potency in IU for the BRP relative to the IS valid at the time of the study (i.e. JNIH-5) but also to the 2nd IS, thereby ensuring continuity.

2. PARTICIPANTS

Fourteen participants from 12 countries within and outside Europe, comprising 6 manufacturers and 8 public institutions involved in the production and/or control of aP-containing vaccines, took part in the joint study. They are listed in section 8 and are referred to herein with a randomly attributed code number, not necessarily linked to the order of listing.

3. MATERIALS, METHODS AND STUDY DESIGN

3.1. Materials

Pertussis toxin BRP (BRP)

The pertussis toxin BRP batch 1 (EDQM catalogue number Y0000021) consists of a freeze-dried preparation of purified pertussis toxin (50 µg/vial). It was calibrated in 2009 against JNIH-5 and assigned a potency of 7500 IU/vial for the HIST [7].

Candidate 2nd International Standard (NIBSC code 15/126)

Purified pertussis toxin was provided to the National Institute for Biological Standards and Control (NIBSC) by the Serum Institute India, formulated and distributed in 1-mL aliquots and then freeze-dried with a final content of approximately 20 µg/ampoule (based on manufacturer's data). The 2nd IS was adopted by the Expert Committee on Biological Standardization (ECBS) in October 2017 with a unitage of 1881 IU/ampoule for HIST and 680 IU/ampoule for the CHO clustering assay [10]. Ampoules of the 2nd IS were kindly provided by NIBSC for the present study.

First WHO International Standard for Pertussis Toxin (JNIH-5)

A freeze-dried purified preparation of pertussis toxin was prepared by the Biken Kanonji Institute in Japan in 1984. This material was reported by the manufacturer to contain 10 µg per ampoule of pertussis toxin by nitrogen content (purity >99.9%). Filling details are as outlined previously [11]. It was assigned 10 000 IU/ampoule for both the lethal HIST and the CHO cell clustering assay through a collaborative study [6]. Ampoules of JNIH-5 were kindly provided by NIBSC for the present study.

3.2. Methods

A standard protocol for the CHO clustering assay, similar to the method used in the BSP114 study [8] for purified PT, was distributed to participants together with standard reporting sheets. Participants were asked to follow the protocol as closely as possible, especially regarding the cell culture medium components, the culture method (thawing and passaging of cells) and seeding density (10 000 to 20 000 cells/well). Cells were to be used between passage numbers 3 and 15 after thawing. Specific instructions for testing the BRP, i.e. dilutions steps, were indicated.

Participants were instructed to reconstitute 1 vial of each material, fresh for each assay, with 1 mL of sterile water for injection, and to discard any unused stock solution at the end of the assay. The reconstituted JNIH-5 had a concentration of 10 000 IU/mL. Once reconstituted, the PT stock solutions were to be kept on ice and used as soon as possible (<1 hour after reconstitution). All further dilutions of the reconstituted PT preparations were done in CHO-K1 culture media consisting of Kaighn's modified Ham's F-12K, 10% foetal bovine serum (FBS), 2 mM L-Glutamine, and an optional antibiotic anti-mycotic solution (Table 1). Providers were suggested in the protocol but participants had the possibility to use reagents from other suppliers provided a comparable grade of purity was ensured and the final composition of the medium was identical. Any deviation from the protocol was to be reported. Assay details, as reported by participants, are summarised in the appendix to this report for information.

Prior to commencing the assays for the study, participants were requested to test their culture system in at least one preliminary assay to verify the sensitivity of the cells, using the BRP provided, and to ensure they were familiar with the protocol.

Acceptance criteria were defined as:

– Cell viability should be at least 95% at passaging;

– A positive score (10 or more cell clusters/well) should be found in at least the first 3 dilutions of the BRP (i.e. 1:150 000 to 1:600 000 dilutions);

– No clusters should be observed in the negative control wells.

Table 1 – *CHO-K1 culture medium ingredients and additional culture reagents*

Component	Suggested Provider, Catalogue number	Volume (mL)
Ham's F-12K medium (Kaighn's modified Ham's F-12 medium)	Life Technologies, 21127 Sigma, N3520 ATCC, 30-2004 Wisent, 312-250 HyClone, SH30526.01	440
Foetal Bovine Serum	**not heat inactivated**: Gibco/Life Technologies, 26040 Invitrogen, 12483 or **heat inactivated**: Tissue Culture Biologics, TCB101HI	50 (final concentration: 10%)
L-Glutamine (200 mM)	Life Technologies, 25030 Sigma, G7513 ATCC, 30-2214 Wisent, 609-065-EL HyClone, SH30034.01	5
Antibiotic-Antimycotic* 100× (Pen G 10 000 U/mL/Strep 10 000 µg/mL/ Amph B 25 µg/mL)	Invitrogen, 15240 Sigma, A5955	5

Additional reagents	Suggested Provider, Catalogue number	Concentration
Trypsin-EDTA	Invitrogen, 25200 Sigma, T4049	0,25%
Trypan blue	Invitrogen, 15250-061 Sigma, T8154	n.a.
Phosphate-Buffered Saline (PBS) pH 7.4	Gibco, 10010	1 ×
Water for Injection	Gibco, A12873 EMD Millipore, 4.86505 Corning, 25-055	n.a.

* The use of antibiotic-antimycotic was optional based on participants' standard cell culture practices

3.3. Study design

If the preliminary assay results were satisfactory, participants were requested to perform three additional independent assays, preferably on different days. Scoring was to be conducted by two individuals trained in observing CHO cell clusters induced by PT, herein referred to as "observers". Results from each observer were reported independently on the appropriate reporting sheets. Each assay included the BRP, JNIH-5 and the 2nd IS. Participants were asked to submit the raw data for each assay so that all data could be analysed using a common method.

4. RESULTS AND STATISTICAL ANALYSIS

Data were provided by 14 participants for the standardised CHO cell clustering assay. In total data from 43 assays were submitted as 13 participants performed 3 assays and 1 participant returned data from 4 assays. However, the first assay was reported as preliminary by this laboratory and thus not included in the calculations. No deviations from the protocol were reported. Raw data from end-point titrations for each of the three provided samples were submitted. Acceptance criteria were assessed as described above (section 3.2). Laboratories 1 and 4 reported results from only 1 observer for one of their assays. Laboratories 3 and 4 reported invalid results for 1 of the observers for 2 of their assays i.e. observation of only 2 positive wells for the BRP instead of the first 3 wells as required in the protocol.

In the case of laboratory 3, only 1 scoring was invalid out of 6 and the other scores were not necessarily at the limit of sensitivity considered in the acceptance criteria (i.e. first 3 BRP wells must be positive). In addition, the plates were prepared from the same cell preparation; therefore, it is very likely that this individual score was an outlier and did not necessarily reflect a problem linked to the cells. It was thus decided to include the valid results of these plates in the calculations. With regard to laboratory 4, two scores were invalid on 2 different plates by 2 different operators. In addition, the other scores were all at the limit of sensitivity. It was thus decided that an issue linked to the cells in this assay could not be excluded and therefore the assay was excluded from the calculations leaving a total of 238 valid end-point determinations for each test sample. The reported end-points (Table 2) did not differ between observers in 32 of 123 dilution series (n = 41 assays, 3 test samples per assay). They were within ± 1 serial dilution in 69 cases, ± 2 serial dilutions in 21 cases and ± 3 serial dilutions in only 1 case. Therefore, the reported end-points did not differ by more than ± 1 serial dilution (1 well difference) between observers and plates within one assay in 82% of the cases.

Relative potency estimates of the BRP and of the 2nd IS to JNIH-5, and of the BRP relative to the 2nd IS, were calculated at the EDQM on the basis of ratios of end-points, where an end-point is the last dilution showing a positive score. Values are given in Table 3 and a graphical representation of the results is depicted in Figure 1 as histograms. Presented are the geometric means (GM) with corresponding geometric coefficient of variation (GCV) per laboratory, as well as GM (1363 and 1361 UI/vial versus JNIH-5 and 2nd IS respectively) and GCV (36% and 33% respectively) across laboratories.

An alternative statistical analysis was also carried out according to Ph. Eur. General Chapter 5.3 on “Statistical analysis of results of biological assays and tests” [12] where the titre was calculated on the basis of ratios of IC50. This led to an estimated potency of 1310 IU/vial. The difference between the titres obtained with the end-point and IC50 calculations can be considered as negligible (less than 5%). IC50 calculations were performed using CombiStats 5.0 [13]. The software automatically applied the Spearman-Kärber method as a result of the very good repeatability of the assay. The number of dilution points with intermediate results, i.e. not equal to either 100% or 0%, was not sufficient to be able to apply the probit method.

Table 2 – *End-point dilutions as reported by participants (given as inverse dilutions divided by 10 000). The statistical analysis was performed centrally.*

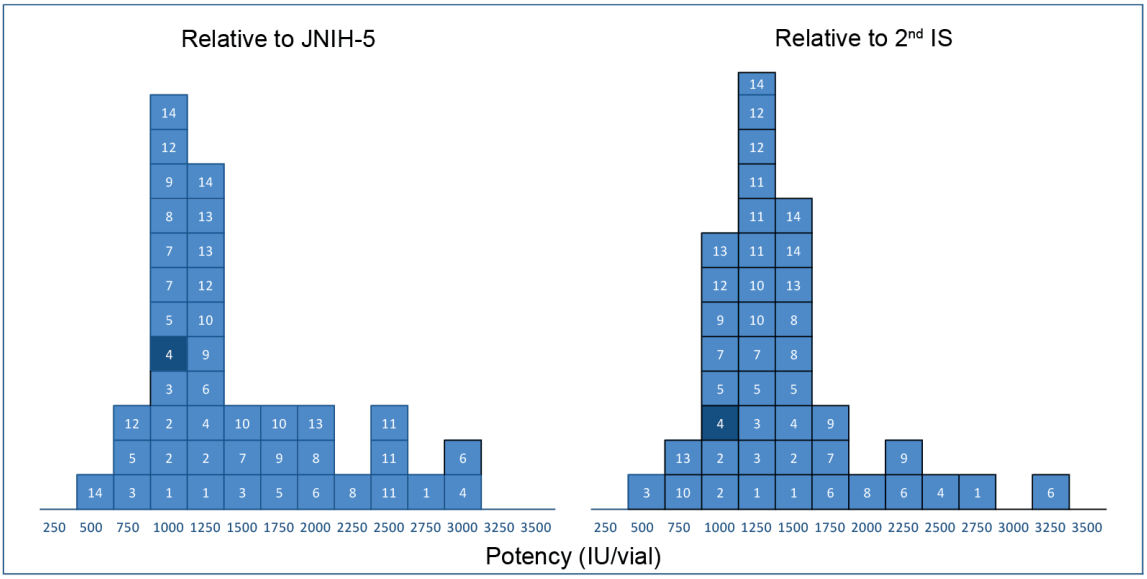
		BRP (dilution/10 000)									2nd IS (dilution/10 000)									JNIH-5 (dilution/10 000)								
		Plate 1		Plate 2		Plate 3					Plate 1		Plate 2		Plate 3					Plate 1		Plate 2		Plate 3				
Lab	Assay	Obs. 1	Obs. 2	Obs. 1	Obs. 2	Obs. 1	Obs. 2	Mean	N	Δ	Obs. 1	Obs. 2	Obs. 1	Obs. 2	Obs. 1	Obs. 2	Mean	N	Δ	Obs. 1	Obs. 2	Obs. 1	Obs. 2	Obs. 1	Obs. 2	Mean	N	Δ
	1	120	n.r.	120	n.r.	120	n.r.	120	3	0	64	n.r.	64	n.r.	64	n.r.	64	3	0	1000	n.r.	2000	n.r.	1000	n.r.	1260	3	1
1	2	240	240	240	480	240	240	269	6	1	64	64	64	64	64	64	64	6	0	1000	500	1000	1000	2000	1000	1000	6	2
	3	240	240	240	240	240	480	269	6	1	128	64	128	64	128	256	114	6	2	2000	2000	2000	1000	4000	2000	2000	6	2
	1	120	60	120	120	120	120	107	6	1	64	64	64	64	128	64	72	6	1	1000	500	1000	1000	2000	1000	1000	6	2
2	2	60	60	60	60	60	120	67	6	1	32	32	64	64	64	64	51	6	1	1000	500	500	1000	500	500	630	6	1
	3	60	60	60	60	120	120	76	6	1	32	64	32	32	32	32	36	6	1	250	500	500	1000	500	1000	561	6	2
	1	60	60	60	60	120	Inv.	69	5	1	32	32	32	64	32	32	36	6	1	500	500	500	500	500	500	500	6	0
3	2	60	120	Inv.	120	60	120	91	5	1	64	256	64	256	64	128	114	6	2	500	4000	500	2000	500	2000	1122	6	3
	3	240	120	120	120	120	120	135	6	1	128	64	128	64	64	64	81	6	1	1000	1000	2000	1000	2000	1000	1260	6	1
	1	60	60	240	60	60	60	76	6	2	32	64	16	32	32	32	32	6	2	500	1000	500	1000	250	500	561	6	2
4	2	120	n.r.	240	n.r.	120	n.r.	151	3	1	64	n.r.	16	n.r.	64	n.r.	40	3	2	500	n.r.	500	n.r.	500	n.r.	500	3	0
	3	60	60	Inv.	60	60	Inv.	n.a.	4	n.a.	32	64	16	128	16	64	n.a.	6	n.a.	500	500	500	500	500	1000	n.a.	6	n.a.
	1	60	240	60	240	120	240	135	6	2	64	64	32	64	32	128	57	6	2	500	500	500	1000	1000	2000	794	6	2
5	2	120	120	120	120	120	120	120	6	0	128	64	128	64	128	64	91	6	1	2000	2000	2000	1000	1000	1000	1414	6	1
	3	240	120	120	120	240	240	170	6	1	128	64	128	128	128	64	102	6	1	2000	2000	1000	2000	1000	2000	1587	6	1
	1	240	240	240	120	120	120	170	6	1	64	64	64	32	64	32	51	6	1	500	1000	500	500	500	500	561	6	1
6	2	240	240	240	240	240	120	214	6	1	32	32	64	64	64	32	45	6	1	1000	2000	1000	1000	1000	1000	1122	6	1
	3	240	120	120	240	240	120	170	6	1	32	64	64	64	64	128	64	6	2	1000	2000	1000	1000	1000	4000	1414	6	2
	1	240	240	120	120	120	240	170	6	1	128	256	128	64	64	128	114	6	2	2000	1000	2000	2000	2000	1000	1587	6	1
7	2	240	240	240	120	120	120	170	6	1	64	128	128	128	64	64	91	6	1	1000	1000	2000	1000	1000	1000	1122	6	1
	3	240	120	120	240	240	240	190	6	1	64	128	64	64	64	64	72	6	1	2000	2000	2000	2000	2000	1000	1782	6	1
	1	120	120	240	120	120	120	135	6	1	64	64	64	64	64	64	64	6	0	2000	1000	2000	1000	1000	1000	1260	6	1
8	2	240	240	240	240	240	240	240	6	0	128	64	128	64	128	128	102	6	1	1000	1000	1000	1000	1000	2000	1122	6	1
	3	120	120	120	120	60	120	107	6	1	32	32	32	64	32	32	36	6	1	500	1000	1000	500	250	500	561	6	2
	1	240	120	120	120	60	120	120	6	2	32	32	32	32	64	32	36	6	1	1000	1000	1000	500	1000	1000	891	6	1
9	2	120	120	120	120	60	60	95	6	1	64	64	64	64	64	64	64	6	0	1000	1000	1000	1000	1000	1000	1000	6	0
	3	120	120	120	120	120	120	120	6	0	64	64	64	32	32	32	45	6	1	1000	1000	500	500	500	1000	707	6	1
	1	240	240	120	120	120	240	170	6	1	64	128	128	128	64	128	102	6	1	1000	2000	1000	1000	1000	1000	1122	6	1
10	2	120	240	240	240	240	240	214	6	1	64	128	128	128	128	256	128	6	2	1000	1000	1000	2000	1000	2000	1260	6	1
	3	240	240	240	120	240	120	190	6	1	128	128	128	128	512	512	203	6	2	2000	2000	4000	1000	1000	1000	1587	6	2
	1	60	60	60	60	60	60	60	6	0	32	32	32	32	32	32	32	6	0	250	250	250	250	250	250	250	6	0
11	2	60	60	60	60	60	60	60	6	0	32	32	32	32	32	32	32	6	0	250	250	250	250	250	250	250	6	0
	3	60	60	60	60	60	60	60	6	0	32	32	32	32	32	32	32	6	0	250	250	250	250	250	250	250	6	0
	1	60	60	60	60	60	60	60	6	0	32	32	32	32	32	32	32	6	0	500	500	500	500	500	500	500	6	0
12	2	60	60	60	60	60	60	60	6	0	32	32	32	32	32	32	32	6	0	1000	1000	500	500	500	500	630	6	1
	3	60	60	60	60	60	60	60	6	0	32	32	64	64	32	32	40	6	1	1000	1000	500	500	1000	1000	794	6	1
	1	120	120	120	120	120	120	120	6	0	64	64	64	64	32	64	57	6	1	500	500	500	1000	500	1000	630	6	1
13	2	60	120	60	120	60	120	85	6	1	64	64	64	64	32	64	57	6	1	500	1000	500	1000	500	1000	707	6	1
	3	60	60	60	60	120	60	67	6	1	64	64	64	64	64	64	64	6	0	500	500	500	500	500	500	500	6	0
	1	120	120	240	120	240	120	151	6	1	64	64	64	64	64	64	64	6	0	4000	4000	4000	4000	4000	4000	4000	6	0
14	2	120	120	120	120	120	120	120	6	0	64	64	64	64	32	32	51	6	1	2000	2000	1000	1000	1000	1000	1260	6	1
	3	240	120	120	120	120	120	135	6	1	128	64	64	128	64	64	81	6	1	1000	1000	2000	1000	1000	1000	1122	6	1
								119									60									878		

Δ: Difference between highest and lowest end-point dilution observed. Shaded cells: invalid assay (excluded from the overall calculation).

Inv.: Invalid; n.a.: not applicable; n.r.: not reported.

Table 3 – *BRP potency relative to the 1st and 2nd IS (IU/vial)*

Lab	BRP relative to JNIH-5				BRP relative to 2nd IS			
	Assay 1	Assay 2	Assay 3	Mean	Assay 1	Assay 2	Assay 3	Mean
1	952	2694	1347	1512	1275	2862	1606	1803
2	1069	1069	1347	1155	1012	902	1431	1093
3	1378	810	1069	1061	1305	542	1136	930
4	1347	3024	Inv.	2018	1606	2550	Inv.	2024
5	1697	849	1069	1155	1606	902	1136	1180
6	3024	1905	1200	1905	2272	3213	1803	2361
7	1069	1512	1069	1200	1012	1275	1803	1325
8	1069	2138	1905	1633	1431	1606	2024	1669
9	1347	952	1697	1296	2272	1012	1803	1606
10	1512	1697	1200	1455	1136	1136	638	937
11	2400	2400	2400	2400	1275	1275	1275	1275
12	1200	952	756	952	1275	1275	1012	1180
13	1905	1200	1347	1455	1431	1012	716	1012
14	378	952	1200	756	1606	1606	1136	1431
	Geometric Mean			1363				1361
	95% Confidence Limits			1140-1630				1153-1607
	GCV (%)			36				33



Numbers are laboratory codes. Cells with a dark background represent one invalid assay. This assay has been excluded from the overall calculations.

Figure 1 – *Histograms of mean (n = 3) potency estimates (IU/vial).*

5. DISCUSSION

The pertussis toxin BRP batch 1 was established in 2000 during the course of an international collaborative study and calibrated in mass units i.e. 50 µg/vial. However, it is known that the biological activity of PT preparations does not always correlate with the mass of protein [6,7,14]. Therefore, the Steering Committee of the Biological Standardisation Programme endorsed a project, BSP076, which led to calibration of the BRP in IU relative to JNIH-5, the 1st WHO IS for PT [7]. According to the European Pharmacopoeia, acellular pertussis vaccine batches need to be tested for residual PT before they can be released onto the market. This has

traditionally been performed by *in vivo* testing using the HIST at the final bulk or final lot level. Alternatively, at the level of purified bulk, an *in vitro* test based on the clustering of CHO cells following exposure to PT can be used to monitor PT presence [3,4]. Until now, this test was not standardised and could not be used in adjuvanted products as the formulation ingredients interfered with the cells in culture. Following a collaborative study (BSP114) investigating a standard method for the CHO clustering assay and a modified method for testing of end-products [8], it was considered necessary to have a reference preparation calibrated in IU for use in this test. The BRP was thus included in a joint EDQM-WHO study in order to allow its calibration in parallel to the establishment of the replacement batch for the 1st WHO IS whose stocks were dwindling. The BRP was included with both other reference preparations in CHO assays performed using the standardised protocol established in BSP114. Since JNIH-5 was originally assigned a potency for the CHO clustering assay in addition to that for the lethal HIST, it was possible to calculate a potency in IU for the BRP for this test.

Potency estimates of the BRP relative to JNIH-5 and to the 2nd IS were calculated centrally at the EDQM (Table 3, Fig. 1). The difference in end-point dilutions between plates and observers within 1 assay was found to be at most ± 1 serial dilution step (1:2 serial dilution steps) in 82% of cases, showing very good intra-assay consistency.

The overall variability is lower than that observed in previous studies [7,8], with overall GCVs of 33 and 36% (Table 3). This shows that stringent application of the standard protocol significantly improves the reproducibility of the assay.

For BSP114, the use of common test samples and a common reference preparation calibrated in IU had been recommended in order to allow comparability between methods and laboratories. The PT BRP was used to this end. However, at the time of the study, the BRP was calibrated solely for the HIST and therefore calculations of CHO cell sensitivity to purified PT or estimations of residual PT in the common vaccine samples were done using the assigned HIST potency, i.e. 7500 IU/vial. This led to sensitivity estimates of around 6-7 mIU/mL for the CHO-K1 cells towards purified PT. In light of the data obtained during the present study, it is clear that the potency measured for the BRP in the CHO clustering assay is different from that in the HIST, in contrast to what was observed for JNIH-5 for which the activity was comparable in both tests [6]. The reasons for this are unclear but it may be postulated that it could be linked to the various regional variants of the HIST used for the calibration, the intrinsic variability of the *in vivo* assay, and/or probably mostly due to the differences in the mode of action of PT in the two tests. The mechanisms underlying the *in vivo* assay remain uncertain despite decades of use of the test. The HIST is subject to complex metabolic phenomena which are unlikely to occur in the cell-based assay. Furthermore, it is unknown whether the relationship between the HIST and the CHO activity levels is linear at all. In addition, a high variability was observed in the non-standardised CHO assays that were used to assign units to JNIH-5. This may also have impacted the CHO unitage assigned to the 1st IS.

Based on the present study data, a potency of 1360 IU/vial is proposed for the BRP for the CHO clustering assay. The calculated overall mean end-point dilution (Table 2) is 1 190 000 for the BRP (n = 238 valid determinations), which corresponds to a concentration of approximately 1.14 mIU/mL. Depending on the sensitivity of the cells and taking account of intrinsic variations due to observers' assessments, a cell sensitivity criterion may be defined for assay validity for routine use. The lowest dilution of BRP showing a positive outcome (10 or more clusters/well) reported by participants during the present study was 1:300 000. This corresponds to 4.5 mIU/mL based on the assigned CHO clustering assay potency. From that, a validity criterion for the test may be proposed, e.g. control wells containing a BRP concentration of 5 mIU/mL or higher should exhibit a positive response (10 or more clusters/well) for the CHO clustering assay to be considered valid. The same criterion may be applied for the confirmation of CHO-K1 cell sensitivity prior to their use in this test.

6. CONCLUSION

Based on the results of the collaborative study, it was thus recommended that the pertussis toxin BRP be assigned a unitage of 1360 IU per vial for the CHO clustering assay. This

recommendation was adopted by the Ph. Eur. Commission in March 2018. The BRP will thus have two different assigned potencies, one for the HIST, i.e. 7500 IU/vial, stemming from the previous calibration exercise [7], and one for the CHO clustering assay as determined by this study. The BRP is available from the EDQM under catalogue number Y0000021. It will be monitored throughout its lifetime through users' trend charts for ethical reasons.

7. ACKNOWLEDGEMENTS

The organisers wish to extend sincere thanks to Dr Kevin Markey (NIBSC, UK) for accepting to be project leader for this arm of the joint study. Thanks go to Ms. Alex Douglas-Bardsley (NIBSC, UK) for organising the collaborative study and providing technical assistance. Thanks also go to all the participants and to their colleagues/co-workers behind the bench for their contribution to the study. Dr Richard Isbrucker (Health Canada, CDN) is warmly thanked for his help with the elaboration of the CHO assay protocol for the study and for his continued support and valuable advice throughout the project and ever since the genesis of the International Working Group for the replacement of the HIST. Ms. Sally Woodward is gratefully acknowledged for her excellent administrative assistance. This study was run in the framework of the Biological Standardisation Programme of the EDQM, a joint programme funded by the European Union and by the Council of Europe, under project code BSP145. This document was produced with financial assistance by the European Union. The views expressed herein can in no way be taken to reflect the official opinion of the European Union.

8. PARTICIPANTS

Thierry Pronce, GlaxoSmithKline Vaccines, BE
Mathias Janssen, Lucas Preux, Fabrice Ribaucour, Scientific Institute of Public Health, BE
Richard Isbrucker, Health Canada, CDN
Meaghan Fowler, Sanofi Pasteur, CDN
Ma Xiao, National Institutes for Food and Drug Control, CN
Pernille Koefoed, Statens Serum Institut, DK
Stéphane Maisonneuve, Agence nationale de sécurité du médicament et des produits de santé, FR
Hildegard Lechner, Paul Ehrlich Institut, DE
Sunil Gairola, Serum Institute of India, IN
Yasuyuki Seki, Biken Kanonji Institute, JP
Joonik Ahn, Ministry of Food and Drug Safety, KR
Marieke Hoonakker, Intravacc, NL
Kevin Markey, National Institute for Biological Standards & Control, UK
Leslie Wagner, Food and Drug Administration, USA

9. ABBREVIATIONS

aP: acellular pertussis; BRP: Biological Reference Preparation; BSP: Biological Standardisation Programme; CHO: Chinese Hamster Ovary cells; DPBS: Dulbecco Phosphate Buffered Saline; ECBS: Expert Committee on Biological Standardization; EDQM: European Directorate for the Quality of Medicines & HealthCare; GCV: Geometric Coefficient of Variation; GM: Geometric Mean; HIST: Histamine Sensitisation Test; IS: International Standard; IU: International Unit(s); NIBSC: National Institute for Biological Standards & Control; Ph. Eur.: European Pharmacopoeia; PT: Pertussis Toxin; WHO: World Health Organization.

10. REFERENCES

[1] Isbrucker R, Arciniega J, McFarland R *et al.* Report on the international workshop on alternatives to the murine histamine sensitization test (HIST) for acellular pertussis vaccines: state of the science and the path forward. *Biologicals* 2014;**42**:114-22.

[2] Hewlett EL, Sauer KT, Myers GA *et al.* Induction of a novel morphological response in Chinese hamster ovary cells by pertussis toxin. *Infect Immun* 1983;**40**:1198-203.

[3] Pertussis vaccine acellular component adsorbed, monograph 1356. Ph. Eur. 9th Edition. Strasbourg, France: Council of Europe; 2016.

[4] Pertussis vaccine acellular co-purified adsorbed, monograph 1595. Ph. Eur. 9th Edition. Strasbourg, France: Council of Europe; 2016.

[5] Gillenius P, Jäätmaa E, Askelöf P *et al.* The standardization of an assay for pertussis toxin and antitoxin in microplate culture of Chinese hamster ovary cells. *J Biol Stand* 1985;**13**:61-66.

[6] Xing D, Gaines Das R, Newland P *et al.* Collaborative study: Evaluation of Proposed International Standard of Pertussis Toxin Code JNIH-5. Ref: BS/03.1978. WHO Expert Committee on Biological Standardization; 2003.

[7] Xing D, Maes A, Behr-Gross ME *et al.* Collaborative study for the standardisation of the histamine sensitizing test in mice and the CHO cell-based assay for the residual toxicity testing of acellular pertussis vaccines. *Pharmeur Bio Sci Notes* 2010;**10**:51-63.

[8] Isbrucker R, Daas A, Wagner L *et al.* Transferability study of CHO cell clustering assays for monitoring of pertussis toxin activity in acellular pertussis vaccines. *Pharmeur Bio Sci Notes* 2016;97-114.

[9] Dobbelaer R, Daas A, Esposito-Farese ME. Collaborative study for the establishment of a European Pharmacopoeia Biological Reference Preparation for Pertussis Toxin – Part 1. *Pharmeur Bio* 2001-1:13-23.

[10] Markey K, Asokanathan C, Tiernay S *et al.* Collaborative study: Evaluation of Proposed Second International Standard for Pertussis Toxin Code: 15/126. Ref: BS/2017.2315. WHO Expert Committee on Biological Standardization; 2017.

[11] Kreeftenberg JG. Collaborative study on the candidate reference materials JNIH-3, JNIH-4 and JNIH-5 for the assay of acellular pertussis vaccines. Ref: BS/88.1586. WHO Expert Committee on Biological Standardization; 1988.

[12] Statistical analysis of results of biological assays and tests, General Chapter 5.3. Ph. Eur. 9th Edition. Strasbourg, France: Council of Europe; 2016.

[13] CombiStats v.5.0, EDQM – Council of Europe. www.combistats.eu.

[14] Miles AA. Biological standards and the measurement of therapeutic activity. British Medical Bulletin 1951;**7**:283-91.

APPENDIX

CHO clustering assay information as reported by participants.

LABORATORY	1	2	3	4	5	6	7
CHO-K1 cell source, catalogue number	ECCAC Sigma, 85051005, batch 09J019	ECACC #85051005	"ATCC CHO-K1, TIB-67"	derived from ATCC CCL61	ATCC CHO-K1, Cat # CCL-61	ECACC	ATCC # CCL-61
Seeding density	30 000 cells/well	20 000 cells/well	10 000 cells/well	20 000 cells/well	12 000 cells/well	20 000 (80 000 cells/mL)	6 × 10^4 cells
Ham's F12K source, catalogue number	Life Technology, 21127-022	Gibco, 21127	Gibco, 21127030	Ham's F-12K (Kaighn's) Medium Gibco, 21127022	Wisent Bioproducts, F-12K Nutrient Mixture (Kaighn's Modification) Cat# 312-250-CL	Sigma #N3520	HyClone, SH30526.01
FBS source, catalogue number	Life Technology, 10270106 batch 42Q5650K	Gibco, 26140	Tissue culture biologics	not heat inactivated GIBCO - Life technologies, 111442FD	Gibco, Cat # 12483-020	Thermo Fisher #26140079	Gibco #10091
L-Glutamine source, catalogue number	Life Technology, 25030-032	Gibco, 25030	Gibco, 25030164	In-house preparation	Gibco, Cat # 25030-081	Sigma, #G7513	Life Technologies, 25030
Antibiotic used	Penicillin - Streptomycin	Pen G 10 000 U/mL/ Strep 10 000 µg/mL/ Amph B 25 ug/ml	Pen-Strep	No antibiotic	100 units/mL of penicillin, 100 µg/mL of streptomycin, and 250 ng/mL of Amphotericin B	Antimycotic (Pen G 10.000 U/ml Strep 10.000 µg/mL Amph B 25 µg/mL) 100x	Pen G 10 000 U/mL/ Strep 10 000 µg/mL
Source antibiotic, catalogue number	Life Technology 15140-148	Gibco, 15240	Gibco 10378016	N/A	Gibco Cat # 15-240-062	Sigma, #A5955	Gibco #15140
Trypsin-EDTA source, catalogue number	Life Technology, 25300-054	Gibco, 25200, 025%	Gibco Trypsin-EDTA Phenol red (0.05%), 25300062	Trypsin-EDTA solution 0.25%, Sigma, T4049-100mL,	0.25% Trypsin-EDTA, Gibco Cat # 25200-056	Sigma, #T4049	Gibco #25200, 0.25%
PBS source, catalogue number	Life Technology, 14190-086	Gibco, 10010	Gibco PBS pH 7.4, 10010031	In-house preparation	Wisent 10× PBS Cat # 311-012-LL	Gibco #10010	Gibco #20012
Water source, catalogue number and pH	In-house sterilized ultrapurified water (MilliQ)	Gibco, A12873	HyClone water for injection (WFI), SH3022117	Lonza, BESP1066B	Cape Cod Associates, LAL Reagent Water, Cat # W020P. pH = 7.2-7.4	Gibco, #A12873*	Invitrogen, 10977, pH7
Experience with CHO assay	yes with whole cell pertussis vaccines	yes	yes	yes for QC of bulk pertussis vaccines	yes	yes for experimental purposes	yes
Comments	assay 1: only 1 observer	None	None	After 4 days confluence of cells was only 90%; trypsinisation 2-3 min without removing trypsin then medium added directly after incubation.	None	pH of water unknown	None

Amph B: amphotericin B; DPBS: Dulbecco's PBS; Pen: penicillin; Strep: streptomycin

LABORATORY	8	9	10	11	12	13	14
CHO-K1 cell source, catalogue number	ECACC	ATCC	ECACC, 85051005	ATCC CCL-61, 61262645	stock maintained in liquid nitrogen	CHO-K1, ATCC CCL-61	ATCC
Seeding density	8 × 10^4 cells/mL	10 000 cells/well	2x 10^4 / 500 µL volume	20 000 cells per well	6 × 10^4 cells/mL suspension; 250 µL/well	assay 1 and 3: 12 500 assay 2: 15 000.	50 000 cells/well
Ham's F12K source, catalogue number	Life Technologies, 21127	Gibco, 21127-022	Sigma, N8641	Gibco Life Technologies, 21127022	Sigma N3520-10L lot SLBQ7914V	Gibco by Life Technologies, 1765029/21765037, Lot: 1737595	Gibco, 2117-022
FBS source, catalogue number	Life Technologies, 10500-064	Gibco, 10099-141	Sigma, F7524	Sigma-Aldrich, F4125-100ml	Moregate (New Zealand) batch B6827103	Life Technologies Europa BV, 10270106, Lot: 42Q3754K	Gibco, 10270-106
L-Glutamine source, catalogue number	Life Technologies, 25030	Gibco, 10378-016	Sigma, G7513	Gibco Life Technologies, 25030081	Sigma G7513-100ml lot. RNBF2231	SSI Diagnostica, 24152, Lot: 241520986	Gibco, 25030-024
Antibiotic used	Antibiotic-antimycotic (pen/strep/Amph B)	Yes	None	100x Antibiotic-antimy-cotic solution	Antibiotic-antimycotic solution stabilised	Gentamicin 2 mg/mL	Penicillin, Streptomycin, Amphotericin B
Source antibiotic, catalogue number	Invitrogen, 15240	Gibco, 15240-062	n/a	Sigma-Aldrich, A5955-100mL	Sigma lot. 105M4823V	SSI Diagnostica, 24180, Lot: 241800986	Gibco, 15240-062
Trypsin-EDTA source, catalogue number	Invitrogen, 25200 0.25%	Gibco, 25200-056 0.25%	Sigma, T4049 (0.25%)	Trypsin-EDTA 0.25% Gibco Life Technologies, 25200056	In-house preparation batch T/16/01, 0.25gm/L (trypsin)	SSI Diagnostica, 24242, Lot. 242420896	Gibco, 25200-056
PBS source, catalogue number	Gibco, 10010	Gibco, 10010-023	In-house (PBS w/o Ca, Mg, pH 7.1)	Gibco Life Technologies, 14190144, DPBS	In-house preparation batch: PBS/25/16	DPBS, calcium, mag-nesium, ThermoFisher SCIENTIFIC, Gibco by Life Technologies, 14040091, Lot. 1708205	Gibco, 20012-019
Water source, cata-logue number and pH	In-house sterilised ultrapurified water (MilliQ)	Gibco, A12873-01	Calbiochem Omnipur, 4.86505.0500 (pH 4.8), Sterile Purified Water (EDQM Sample Code 55513)	OmniPur, 7732-18-5/4.86505	In-house batch: 0675460017	Statens Serum Institut, FM046, Lot: 371530, pH 6,07	Gibco, A1273-01
Experience with CHO assay	Yes	Yes	yes (participation in BSP114 phase 2 study; assay currently under development for routine use in vaccine batch release)	Yes	Yes	The laboratory where the analysis was performed does not have any previous experience with a CHO clustering assay.	Yes, BSP114 and validation of routine test
Comments	None	None	None	Reagent deviations: 1. FBS - Sigma # F4135 2. DPBS- Gibco Life Technologies, 14190144 Method deviations: 1. Cells grown in 10 mL of CGM in T75 flask 2. 1 mL of Trypsin-EDTA	None		None

Amph B: amphotericin B; DPBS: Dulbecco's PBS; Pen: penicillin; Strep: streptomycin

ENG

www.edqm.eu

The Council of Europe is the continent's leading human rights organisation. It comprises 47 member states, including all members of the European Union. The European Directorate for the Quality of Medicines & HealthCare (EDQM) is a directorate of the Council of Europe. Its mission is to contribute to the basic human right of access to good quality medicines and healthcare and to promote and protect public health.

ISSN: 2075-2504

edqm
European Directorate for the Quality of Medicines & HealthCare | Direction européenne de la qualité du médicament & soins de santé

COUNCIL OF EUROPE
CONSEIL DE L'EUROPE