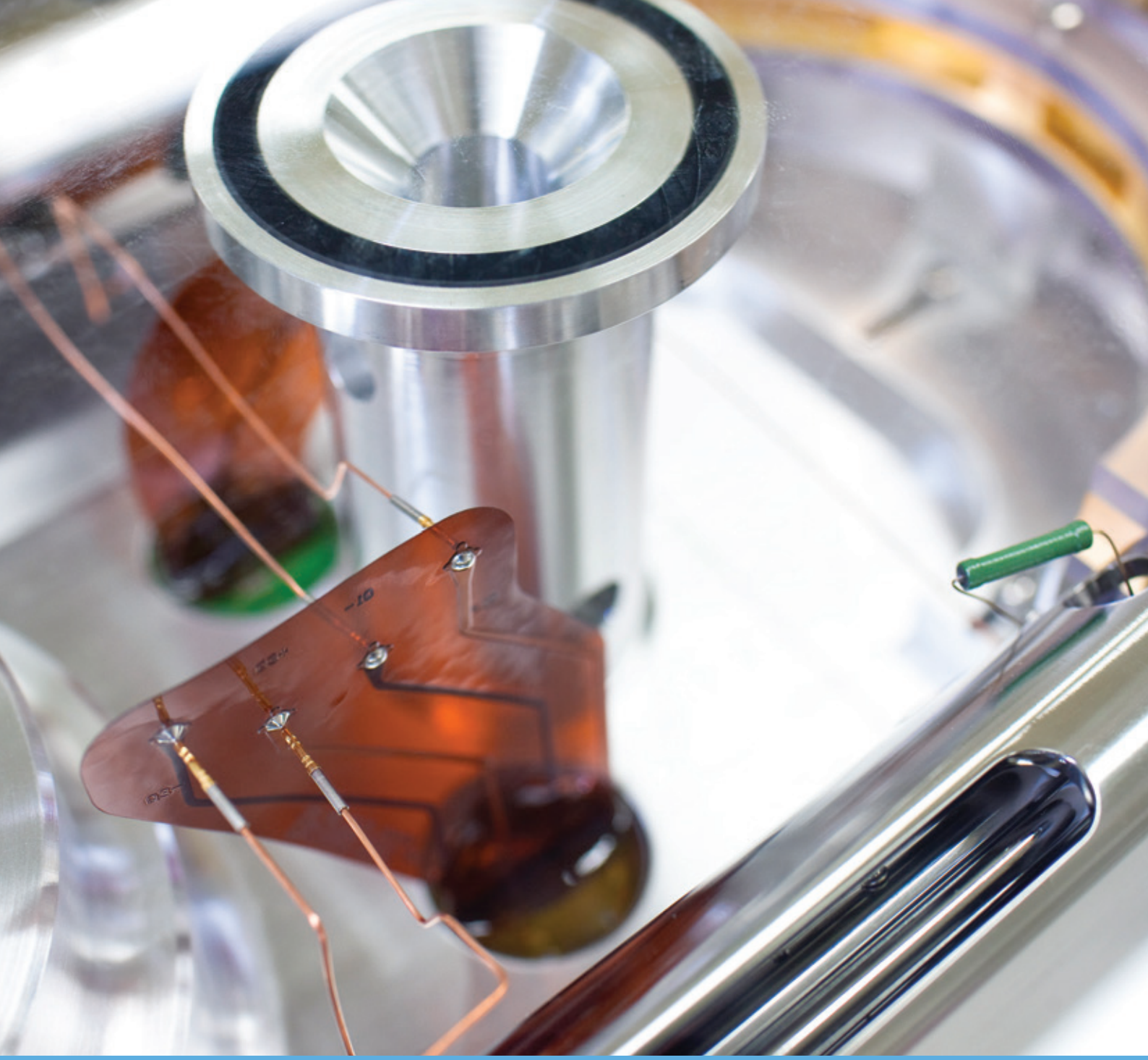

Determination of pentachlorophenol in feed materials and compound feed by LC-MS/MS

R.J.B. Peters and W. Gebbink

WAGENINGEN
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This research has been carried out by Wageningen Food Safety Research, institute within the legal entity Wageningen Research Foundation funded by The European Committee for Standardization, CEN, project number 1247320701.

Wageningen, June 2019

WFSR report 2019.009

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Contaminant expert: R.J.B. Peters

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WFSR report 2019.009

Distribution list:

- 21 participating laboratories of which 14 European participants, 6 Asian participants and 1 North-American participant.

Contents

Summary

European Commission Mandate M/523 called for the standardisation for a method for the pentachlorophenol (PCP) analysis in compound feed and feed materials (including guar gum and fatty acid distillates). WFSR (at that time RIKILT) Wageningen University & Research was requested by the European Committee for Standardization (CEN) to develop the standard. An LC-MS/MS method was developed for the determination of PCP in compound feed, guar gum and fatty acid distillates with a limit of quantitation of 10 µg/kg. For the developed standard a collaborative trial was organised between February and April 2018 for the validation of the standard.

Twenty-one laboratories subscribed for participation in this test and sixteen reported results. The analysis of PCP in these matrices showed HorRat values from 0.39 to 2.21. The analysis of PCP in Feed 2 resulted in a HorRat value above the accepted value of 2 (2.21) while the analysis of PCP in the other samples resulted in HorRat values below the accepted value of 2. The presence of possible interferences in Feed 2 could have impacted the results submitted by the participants, although this was not observed during homogeneity testing. Overall, it can be concluded that the prescribed method 'CEN/TC 327 for the determination of PCP in feed materials and compound feed by LC-MS/MS' is fit-for-purpose.

1 Introduction

European Commission Mandate M/523 called for the standardisation for a method for the pentachlorophenol (PCP) analysis in compound feed and feed materials (including guar gum and fatty acid distillates). WFSR (at that time RIKILT) Wageningen University & Research was requested by the European Committee for Standardization (CEN) to develop the standard. An LC-MS/MS method was developed for the determination of PCP in compound feed, guar gum and fatty acid distillates with a limit of quantitation of 10 µg/kg. For the developed standard a collaborative trial was organised between February and April 2018 for the validation of the standard. We here report on the results of this collaborative trial.

2 Participants

Twenty-one laboratories registered for participation in the validation study. Fourteen of them are situated in Europe, six in Asia and one in North America. Sixteen of these submitted results; some labs did not get the samples through customs clearance, others were unable to report results.

3 Design of the study

3.1 Sample preparation

Five samples (Table 1) were prepared: two feed samples, one guar gum sample and two fatty acid distillate (FAD) samples. The samples were prepared by RIKILT Wageningen University & Research, The Netherlands. The feed and FAD samples were compiled from different ingredients; the guar gum was one batch, received as an import sample. Because none of the samples contained any 'natural' contamination with pentachlorophenol the samples were fortified at the desired levels. The ingredients together with the pentachlorophenol solution were mixed thoroughly and the 5 samples were stored in suitable containers. The codes and aimed levels of the materials are presented in Table 1.

Table 1 *Samples of the method validation study*

Material	Code	Aimed level (µg/kg)
Compound feed 1	A and B	8
Compound feed 2	C and D	13
Guar gum	E and F	15
Fatty acid distillate 1	G and H	10
Fatty acid distillate 2	I and J	20

3.2 Sample identification

After homogenization, the feed materials were divided into sub-portions of 6 gram and the guar gum and FAD samples into portions of 3 gram. The samples for the participants were randomly coded by a web application (Annex 1). Per material 10 randomly taken samples were used for homogeneity testing.

3.3 Homogeneity study

The homogeneity of all materials was tested according to The International Harmonized Protocol for Proficiency Testing of Analytical Laboratories and ISO 13528, taking into account the insights discussed by Thompson [1] regarding the Horwitz equation. Ten containers of each material were analysed in duplicate for PCP. The results are presented in Annex 2. All materials were considered as homogeneous after statistical evaluation.

3.4 Sample distribution and instructions

Each of the participating laboratories received a randomly assigned laboratory code, generated by a web application, designed for proficiency tests. The sample sets, consisting of ten coded samples (two samples of each material, Annex 1) and vials containing PCP and its ^{13}C mass labelled analogue, were sent to the participating laboratories on February 19, 2018. The sample sets were packed in a carton box and were dispatched to the participants immediately by courier.

The samples were accompanied by a letter (Annex 3) describing the requested analyses and an acknowledgement of receipt form. By e-mail the laboratories received instructions on how to use the web application for reporting results.

The laboratories were asked to store the samples in the refrigerator until analysis. A single analysis of each sample was requested using the prescribed method '*Determination of pentachlorophenol (PCP) in feed materials and compound feed by LC-MS/MS*' (Annex 4). The deadline for submitting the results was April 13, 2018, allowing 8 weeks for the analysis.

Several laboratories reported about small modifications to the prescribed method. These were communicated with the expert beforehand or were reported with the submission of the results:

PT092: different LC column and mobile phase were used compared to the prescribed method. The column used by the participant was a C18 column, only with different dimension (1.8um, 150x2.1mm). The mobile phase contained ammonium acetate instead of ammonium formate as described in the method. Both changed were estimated to have no impact on the results of the participant.

PT142: final extract were filtered using a 0.2 µm filter, this step was not included in the prescribed method. This additional step is estimated to have no impact on the results of the participant.

PT143 & PT145: different LC-MS parameters were used compared to the prescribed method. As mass spectrometers were used from different companies, certain parameters need to be optimised and will therefore be different compared to the prescribed method. These changes were estimated to have no impact on the results from the participants.

PT151: a different sample amount was analysed for the guar gum samples. The participant analysed the whole vial containing approximately 3 gram guar gum instead of the prescribed 1.0 gram. As the exact sample weight was not recorded by the participant, a concentration could not be calculated. The submitted results of the guar gum samples (reported as an amount) were therefore removed.

4 Statistical evaluation

The main objective of this collaborative study is the validation of the new candidate CEN method for the determination of PCP in feed materials and compound feed.

Statistical evaluation of the results was carried out according robust statistics according to ISO 5725 [2]. As some participants submitted single values for a given sample and not duplicate values, these results were removed from the dataset prior to the statistical evaluation. In order to identify irregular data Mandel h and k plots were generated, and irregular data was removed from the dataset. After evaluation of the data, the HorRat (Horwitz ratio) is calculated. HorRat is described for evaluation of collaborative studies. The HorRat is the ratio of the reproducibility relative standard deviation (expressed as a percentage (RSD_R %)) to the predicted reproducibility relative standard deviation (expressed as a percentage (RSD_R %)):

$$\text{HorRat} = \frac{\text{RSD}_R(\%)}{\text{PRSD}_R(\%)}$$

The $PRSD_R$ (%) is calculated using the Horwitz equation. However, this equation is not applicable to the lower concentration range (<120 µg/kg) [1]. Therefore a $PRSD_R$ (%) of 22% is used in this study (corresponding to the value when 120 µg/kg is entered in the Horwitz equation). The acceptable value for the HorRat value is <2.0.

An overview of the reported concentrations is presented in Annex 5, and results were excluded based on the Mandel h and k plots (Annex 6).

The final results show HorRat values <2 for feed 1, guar gum and FAD 1 & 2 ranging from 0.39 in guar gum to 1.36 in FAD 1 (Table 2). For feed 2 an HorRat value 2.21 was obtained, and is therefore above the accepted value above 2.

Table 2 *PCP - Results summary of the collaborative trial*

Material name	Feed 1	Feed 2	Guar gum	FAD 1	FAD 2
Analyte	PCP	PCP	PCP	PCP	PCP
Year of inter-laboratory study	2017/18	2017/18	2017/18	2017/18	2017/18
Number of laboratories	13	14	14	13	14
Number of samples	2	2	2	2	2
Number of laboratories considered as non-compliant	1	1	0	1	2
Number of accepted results	24	26	28	24	24
Mean value, x a, µg/kg	8.4	12.8	14.6	12.2	23.5
Repeatability standard deviation s_r, µg/kg	0.7	2.9	1.0	2.7	3.6
Repeatability relative standard deviation, RSD_r, %	8.4	23.0	6.7	21.8	15.3
Repeatability limit r [r = 2,8 x s_r], µg/kg	2.0	8.3	2.7	7.5	10.1
Reproducibility standard deviation s_R, µg/kg	2.1	6.2	1.3	3.6	3.6
Reproducibility relative standard deviation, RSD_R, %	24.6	48.7	8.7	29.9	15.3
HorRat value	1.12	2.21	0.39	1.36	0.70
Reproducibility limit R [R = 2,8 x s_R], µg/kg	5.8	17.5	3.5	10.2	10.1

5 Conclusion

Within this collaborative study for PCP in feed, guar gum and fatty acid distillate, the prescribed method was tested by sixteen laboratories worldwide.

The analysis of PCP in these matrices showed HorRat values from 0.39 to 2.21. The analysis of PCP in Feed 2 resulted in a HorRat value above the accepted value of 2 (2.21). The grand mean for Feed 2 was 12.8 µg/kg, whereas the performance in Feed 1, with a lower grand mean of 8.4 µg/kg, was satisfactory (HorRat value of 1.12). Feed 1 and 2 were composed of different compound feed samples. The presence of possible interferences in Feed 2 could have impacted the results submitted by the participants, although this was not observed during homogeneity testing.

Overall, it can be concluded that the prescribed method 'CEN/TC 327 for the determination of PCP in feed materials and compound feed by LC-MS/MS' is fit-for-purpose.

References

1 Thompson M. 2000. Recent trends in inter-laboratory precision at µg/l and sub-µg/l concentrations in relation to fitness for purpose criteria in proficiency testing. Analyst. 125: 385-386.

2 ISO 5725:1994 Accuracy (trueness and precision) of measurement methods and results.

Annex 1 Codification of the samples

	material A	material B	material C	material D	material E	material F	material G	material H	material I	material J
Labcode	feed 1		feed 2		guar gum		FAD 1		FAD 2	
PT084	977	811	131	476	492	765	433	452	417	528
PT092	497	545	910	677	236	160	414	646	251	736
PT141	981	512	644	891	432	841	204	618	138	982
PT142	725	481	919	217	654	535	628	809	496	812
PT143	607	986	683	979	598	430	688	113	170	566
PT144	956	254	998	331	356	732	346	242	182	815
PT145	735	471	214	442	325	701	253	273	116	461
PT146	266	764	234	904	513	241	122	270	883	257
PT147	926	466	779	453	151	199	734	381	522	599
PT148	178	623	834	102	483	854	255	388	954	410
PT149	572	472	791	440	879	948	464	951	305	756
PT150	120	349	362	718	172	766	738	827	762	171
PT151	237	508	840	403	343	825	817	449	144	932
PT152	438	749	997	175	931	164	511	851	458	147
PT153	789	446	604	215	722	196	180	399	814	498
PT154	798	988	126	577	673	173	244	450	676	493
PT155	697	740	679	206	945	633	127	103	262	519
PT156	606	416	588	533	964	934	319	705	829	408
PT157	184	250	699	957	838	288	579	209	397	167
PT158	571	818	556	613	114	675	287	322	478	564
PT159	630	469	150	546	221	531	473	157	617	299

Annex 2 Homogeneity

PCP in feed 1 (AB, µg/kg)		
Replicate 2	Replicate 1	Replicate 2
Hom/AB001	8.1	8.4
Hom/AB002	7.5	9.0
Hom/AB003	9.7	9.0
Hom/AB004	8.0	10.0
Hom/AB005	8.9	8.3
Hom/AB006	8.3	9.2
Hom/AB007	9.6	9.4
Hom/AB008	9.5	9.9
Hom/AB009	9.4	9.6
Hom/AB010	10.4	10.0
Grand mean	9.1	
Cochran's test		
C	0.450	
Ccrit	0.602	
C<Ccrit?	NO OUTLIERS	
Target s = σ_H	2.00	
S_r	0.64	
S_w	0.65	
S_s	0.46	
Critical = 0.3σ_H	0.60	
S_s<critical?	ACCEPTED	
S_w<0.5σ_H?	ACCEPTED	

PCP in feed 2 (CD, µg/kg)		
Replicate 2	Replicate 1	Replicate 2
Hom/CD001	14.2	14.3
Hom/CD002	14.2	14.8
Hom/CD003	13.8	13.0
Hom/CD004	12.6	14.0
Hom/CD005	12.1	12.9
Hom/CD006	14.9	12.0
Hom/CD007	12.8	14.9
Hom/CD008	13.3	11.7
Hom/CD009	13.8	14.4
Hom/CD010	14.0	12.7
Grand mean	13.5	
Cochran's test		
C	0.405	
Ccrit	0.602	
C<Ccrit?	NO OUTLIERS	
Target s = σ_H	2.98	
S_r	0.67	
S_w	1.04	
S_s	0.00	
Critical = 0.3σ_H	0.89	
S_s<critical?	ACCEPTED	
S_w<0.5σ_H?	ACCEPTED	

PCP in guar gum (EF, µg/kg)		
Replicate 2	Replicate 1	Replicate 2
Hom/EF001	14.8	15.6
Hom/EF002	14.3	14.1
Hom/EF003	15.9	13.9
Hom/EF004	14.9	14.7
Hom/EF005	14.2	14.8
Hom/EF006	15.3	14.4
Hom/EF007	15.4	14.8
Hom/EF008	16.5	14.7
Hom/EF009	13.6	13.7
Hom/EF010	14.7	14.9
Grand mean	14.8	
Cochran's test		
C	0.415	
Ccrit	0.602	
C<Ccrit?	NO OUTLIERS	
Target s = σ_H	3.25	
S_r	0.55	
S_w	0.68	
S_s	0.27	
Critical = 0.3σ_H	0.97	
S_r<critical?	ACCEPTED	
S_w<0.5σ_H?	ACCEPTED	

PCP in FAD 1 (GH, µg/kg)		
Replicate 2	Replicate 1	Replicate 2
Hom/GH001	12.5	11.7
Hom/GH002	11.2	11.7
Hom/GH003	11.6	12.0
Hom/GH004	11.7	11.7
Hom/GH005	10.6	11.3
Hom/GH006	10.6	11.3
Hom/GH007	10.2	10.2
Hom/GH008	Outlier	Outlier
Hom/GH009	10.2	10.9
Hom/GH010	11.2	10.9
Grand mean	11.2	
Cochran's test		
C	0.268	
Ccrit	0.638	
C<Ccrit?	NO OUTLIERS	
Target s = σ_H	2.46	
S_r	0.61	
S_w	0.37	
S_s	0.55	
Critical = 0.3σ_H	0.74	
S_r<critical?	ACCEPTED	
S_w<0.5σ_H?	ACCEPTED	

PCP in FAD 2 (IJ, µg/kg)		
Replicate 2	Replicate 1	Replicate 2
Hom/IJ001	21.0	21.9
Hom/IJ002	23.3	23.0
Hom/IJ003	22.1	21.8
Hom/IJ004	19.9	22.4
Hom/IJ005	22.7	21.6
Hom/IJ006	22.1	22.8
Hom/IJ007	19.5	22.3
Hom/IJ008	22.1	22.2
Hom/IJ009	23.0	23.3
Hom/IJ010	21.5	23.7
Grand mean	22.1	
Cochran's test		
C	0.355	
Ccrit	0.638	
C<Ccrit?	NO OUTLIERS	
Target s = σ_H	4.87	
S_s	0.78	
S_w	1.04	
S_s	0.27	
Critical = 0.3σ_H	1.46	
S_s<critical?	ACCEPTED	
S_w<0.5σ_H?	ACCEPTED	

Annex 3 Instruction letter

WAGENINGEN
UNIVERSITY & RESEARCH

P.O. Box 230 | 6700 AE WAGENINGEN | The Netherlands

RIKILT

DATE
February 12, 2018

SUBJECT
analysis of pentachlorophenol

OUR REFERENCE
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Ir. I.J.W. Elbers

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Dear participant,

Thank you very much for your interest in the collaborative study for the analysis of pentachlorophenol (PCP) by LC-MS/MS in feed materials and compound feed. Hereby I send you a parcel containing ten tubes containing compound feed, guar gum or fatty acid distillate and two vials containing standard solution of PCP and of $^{13}C_6$-PCP.

Please fill out the accompanied 'acknowledgement of receipt form' and return it immediately upon receipt of the samples, preferably by e-mail.
Instructions:

- After arrival store the samples at +4°C.
- Please analyze the samples according to the prescribed method *"Animal feed – Determination of pentachlorphenol (PCP) in feed materials and compound feed"*. Please closely adhere to this protocol. Small changes should be documented. Substantial changes should be agreed upon beforehand with Wouter Gebbink (Wouter.Gebbink@wur.nl).
- The deadline for reporting your results is **April 13, 2018.**
- Please use the web application for entering your results (https://crlwebshop.wur.nl/apex/f?p=307:LOGIN).

- Your username is:
- Your password is:
- Your lab code to enter this collaborative study is:

With kind regards,

Ingrid Elbers
Wouter Gebbink
RIKILT Wageningen University & Research
Netherlands

Annex 4 Method description

CEN/TC 327

Date: 2018-07

prEN XXXXX: XXXX

Secretariat: NEN

Determination of pentachlorophenol (PCP) in feed materials and compound feed by LC-MS/MS

ICS:

CCMC will prepare and attach the official title page.

Contents

Page

European foreword

This document (prEN XXXX:XXXX) has been prepared by Technical Committee CEN/TC 327 "Animal feeding stuffs", the secretariat of which is held by NEN.

This document is currently submitted to the CEN Enquiry.

This document will supersede EN XXXX:XXXX.

This document has been prepared under a mandate given to CEN by the European Commission and the European Free Trade Association, and supports essential requirements of EU Directive(s).

For relationship with EU Directive(s), see informative Annex ZA, which is an integral part of this document.

Introduction

European Commission Mandate M/523 called for the standardisation for a method for the pentachlorophenol (PCP) analysis in compound feed and feed materials (including guar gum and fatty acid distillates). RIKILT Wageningen University and Research was requested by the European Committee for Standardization (CEN) to develop the standard.

1 Scope

This European Standard specifies a liquid chromatographic method with triple-quadrupole mass spectrometry (MS/MS) detection for the determination of pentachlorophenol (PCP) in feed materials and animal feed.

The limit of quantitation (LOQ) for the PCP determination in guar gum, fatty acid distillates (FAD) and animal feed is 10 µg/kg. Individual laboratories are responsible for ensuring that the equipment that they use will achieve this limit of quantification.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN ISO 6498, *Animal feeding stuffs – Preparation of test samples (ISO 6498)*

3 Terms and definitions

No terms and definitions are listed in this document.

4 Principle

In order to check for the presence of PCP, a test portion of sample material is fortified with internal standards (^{13}C-PCP) and is extracted using a QuEChERS approach for animal feed and FAD, and a 'reversed' QuEChERS approach for guar gum. For FAD samples, lipids are removed prior to QuEChERS extraction by the addition of sulfuric acid. Final extracts from all matrices are analysed by liquid chromatography coupled to triple quadrupole mass spectrometry, operated in negative electrospray ionization mode. Identification is done on the basis of retention time and mass. Quantification is done using the internal standard method.

5 Reagents and materials

5.1 General

Use only reagents of recognized analytical grade and with a purity suitable for OC residue analysis. Check the purity of the reagents by performing a blank test under the same conditions as used in the method. The chromatogram should not show any interfering impurity at the retention time of compounds of interest.

5.2 Chemicals

5.2.1 Acetonitrile

5.2.2 Methanol

5.2.3 Acetone

5.2.4 n-Hexane

5.2.5 Sulfuric acid

5.2.6 Deionized water

5.2.7 Sodium hydroxide

5.2.7.1 Sodium hydroxide solution (10 M)

Weigh 4 g of sodium hydroxide (5.2.7.1) and add 10 ml deionized water (5.2.6) to achieve a concentration of 10 M. Store at room temperature in a closed glass bottle.

5.2.8 Magnesium sulphate

5.2.9 Sodium chloride

5.2.10 Formic acid

5.2.11 Ammonium formate

5.2.11.1 Ammonium formate solution (1M)

Weight 6.3 g ammonium formate (5.2.11) and add 100 mL deionized water (5.2.6) to achieve a concentration of 1 M. Store the solution at room temperature. The solution is tenable under these conditions during at least 1 month.

5.2.12 Mobile phase A

Take 1 mL ammonium formate solution (1 M) (5.2.11.1) and add 999 mL deionized water (5.2.6) and 20 µL formic acid (5.2.10) and mix well. Store the solution at room temperature. The solution is tenable under these conditions during at least 1 month.

5.2.13 Mobile phase B

Take 1 mL ammonium formate solution (1 M) (5.2.11.1) and add 50 mL deionized water (5.2.6), 949 mL methanol (5.2.2) and 20 µL formic acid (5.2.10) and mix well. Store the solution at room temperature. The solution is tenable under these conditions during at least 1 month.

5.2.14 Pentachlorophenol (PCP sodium salt, 95% purity)

5.2.14.1 PCP Stock solution 1 (2000 µg/ml)

Weigh 25 mg (± 0.01 mg) of PCP (5.2.14) (taking impurities into consideration) and add 12.5 mL deionized water (5.2.6) to achieve a concentration of 2000 µg/ml. Store the solution in a refrigerator at 4°C (± 3°C). The solution is tenable under these conditions during at least 12 months.

5.2.14.2 PCP Stock solution 2 (1 ng/µl)

Dilute 50 µL of Stock solution 1 (5.2.14.1) to 100 mL deionized water (5.2.6) in a volumetric flask (5.2.20). Store the solution in a refrigerator at 4°C (± 3°C). The solution is tenable under these conditions during at least 12 months.

5.2.14.3 PCP Working solution 1 (0.1 ng/µl)

Take 100 µL of Stock solution 2 (5.2.14.2) and add 900 µL acetonitrile (5.2.1). prepare this solution daily.

5.2.14.4 PCP Working solution 2 (0.01 ng/µl)

Take 50 µL of Working solution 1 (5.2.14.3) and add 450 µL acetonitrile (5.2.1). prepare this solution daily.

5.2.15 $^{13}C_6$-pentachlorofenol of certified purity (> 99%, 100 ng/µL) as an internal standard

5.2.15.1 $^{13}C_6$-PCP Stock solution (10 ng/µl)

Take 1 mL of $^{13}C_6$-PCP at 100 ng/µL (5.2.15) and dilute with acetone (5.2.3) to 10 mL in a volumetric flask (5.2.19) and mix well. Store the solution in a refrigerator at 4°C (± 3°C).

5.2.15.2 $^{13}C_6$-PCP Working solution 1 (1 ng/µl)

Take 100 µL of Stock solution (5.2.15.1) and add 900 µL acetonitrile (5.2.1). prepare this solution daily.

5.2.15.3 $^{13}C_6$-PCP Working solution 2 (0.1 ng/µl)

Take 50 µL of Working solution 1 (5.2.15.2) and add 450 µL acetonitrile (5.2.1). prepare this solution daily.

5.2.16 Calibration standards

Prepare calibration mixtures according to Table 1 in a final volume of 1.0 mL of acetonitrile and store them at 4°C (± 3°C).

Table 1. Calibration mixtures

Level	Concentration [ng/mL]	PCP 0.01 ng/µL (5.2.14.4)	PCP 0.1 ng/µL (5.2.14.3)	PCP 1 ng/µL (5.2.14.2)	Internal standard 0.1 ng/µL (5.2.15.3)	ACN (5.2.1)
1	0				20 µL	980 µL
2	0.1	10 µL			20 µL	970 µL
3	0.5	50 µL			20 µL	930 µL
4	1.0		10 µL		20 µL	970 µL
5	5.0		50 µL		20 µL	930 µL
6	10		100 µL		20 µL	880 µL
7	40			40 µL	20 µL	940 µL

5.2.17 PP screwcap centrifuge tube (Greiner), 50 mL

5.2.18 Glass tube with screw cap, 12 mL

5.2.19 Volumetric flask, 10 mL

5.2.20 Volumetric flask, 100 mL

5.2.21 LC-MS vial, 2 mL

6 Apparatus

6.1 Analytical balance, accuracy to 0.1 mg

6.2 Centrifuge

6.3 Water bath

6.4 Liquid chromatograph connected to a triple-quadrupole mass spectrometer

6.4.1 LC conditions

The liquid chromatograph is equipped with a Symmetry C18 column (5 µm, 3.0 × 150 mm) (or comparable column). The flow of the mobile phase is kept constant at 500 µl/min, while the column is kept at 40 °C. The injection volume is 20 µL.

The gradient program of mobile phases A and B (5.2.12 and 5.2.13, respectively) are mentioned in Table 2.

Table 2. Mobile phase gradient for LC-MSMS analysis

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Flow (µl/min)
0	55	45	500
1	55	45	500
4	8	92	500
9	8	92	500
9.5	55	45	500
16	55	45	500

6.4.2 MS conditions

The mass-spectrometer shall be capable of Multiple Reaction Monitoring (MRM), and should be tuned according to the manufacturer's description. In MS/MS mode, no fragmentation of PCP was observed at the highest collision energy setting. However, a MRM transition channel was used with minimal collision

energy using the same masses for precursor and product ion selection in order to reduce background noise.

The following parameters were used:

Table 3. MS parameters

Source	
ESI	negative
Capillary (kV)	3.50
Cone (V)	30
Source temperature (°C)	120
Desolvation temperature (°C)	350
Cone gas flow (L/Hr)	200
Desolvation gas flow (L/Hr)	600

Analyser	
LM 1 resolution	12.5
HM 1 resolution	12.5
Ion energy 1	1.0
LM 2 resolution	12.5
HM 2 resolution	12.5
Ion energy 2	1
Multiplier	750

NOTE: these settings should be optimized for the used instrument, the shown values are indicative.

The selected mass fragments are shown in Table 4. At least one quantifier transition and one qualifier transition have to be measured.

Table 4. MRM transitions for PCP

Analyte	MRM Transition Quantifier	MRM Transition Qualifier	Dwell time (s)	Cone (V)*	Collision energy (eV)*
PCP	262.8>262.8	264.8>264.8	0.2	30	7
$^{13}C_6$-PCP	272.8>272.8	274.8>274.8	0.2	30	7

* Cone voltages and collision energies should be optimized for the used instrument, the shown values are indicative.

7 Sampling

The sample should be truly representative and not been damaged or changed during transport or storage. Sampling is not part of the method specified in this European Standard. A recommended sampling method is given in ISO 6497 [1].

8 Preparation of the test sample

Prepare the test sample in accordance with EN ISO 6498 [2].

Dry or low moisture products such as cereals and cereal products, mixed feeds, and hay should be ground carefully so that it passes completely through a sieve with 1 mm apertures. Mix thoroughly.

9 Procedure

9.1 General

Analyse in each series the following samples:

1) Procedural blank;
2) Blank matrix (n=1);
3) Blank matrix spiked (n=2);
4) All samples.

NOTE matrix could be guar gum, fatty acid distillate and/or animal feed. Any blank matrix sample proven to be blank in a previous run can be used for quality control.

9.2 Test portions and Extraction

9.2.1 Guar gum

- Weigh 1.0 g (± 0.05 g) of the prepared test sample into a 50 ml PP screwcap centrifuge tube (Greiner) (5.2.17).
- Add 10 ml Acetonitrile (5.2.1) and shake immediately for 10 seconds.
- Spike the sample with 20 µL $^{13}C_6$-PCP Working solution 1 (1 ng/µl) (5.2.15.2).
- Add 10 ml deionized water (5.2.6) and shake for 1 minute.
- Add the pre-weight salts [4 gram Magnesium Sulphate (5.2.8) and 1 gram Sodium chloride (5.2.9)], shake immediately for 1 minute.
- Centrifuge for 5 minutes at 3500 rpm.
- Transfer 1 ml acetonitrile phase in a LC-MS vial (5.2.21).

9.2.2 Animal feed

- For a wet animal feed weigh 10.0 g (± 0.10 g) of the prepared test sample into a 50 ml PP screwcap centrifuge tube (Greiner) (5.2.17), and spike with 20 µL $^{13}C_6$-PCP Working solution 1 (1 ng/µl) (5.2.15.2) and let it stand for at least 1 hour.
- For a dry animal feed weigh 2.5 g (± 0.10 g) of the prepared test sample into a 50 ml PP screwcap centrifuge tube (Greiner) (5.2.17) and spike with 20 µL $^{13}C_6$-PCP Working solution 1 (1 ng/µl) (5.2.15.2), add 7,5 ml deionized water (5.2.6), and let it stand for at least 1 hour.
- Add 10 ml Acetonitrile (5.2.1) and shake for 1 minute.

- Add the pre-weight salts [4 gram Magnesium Sulphate (5.2.8) and 1 gram Sodium chloride (5.2.9)], shake immediately powerful for 1 minute.
- Centrifuge for 5 minutes at 3500 rpm.
- Transfer 1 ml acetonitrile phase in a LC-MS vial (5.2.21).

9.2.3 Fatty acid distillate

- Weigh 1.0 g (± 0.05 g) of the prepared test sample into a 12 ml glass tube with screwcap (teflon inlay) (5.2.18).
- Spike the sample with 20 µl $^{13}C_6$-PCP Working solution 1 (1 ng/µl) (5.2.15.2).
- Add 5 ml of hexane (5.2.4) and mix until the sample is dissolved.
- Add 1 ml concentrated sulfuric acid (5.2.5). Place the tubes during 30 minutes in a water bath at 60 °C, mix carefully every 10 minutes.
- Leave the samples to cool to room temperature and centrifuge for 5 minutes at 2000 rpm.
- Transfer the organic layer to a clean glass tube (5.2.18).
- Repeat the extraction of the sulphuric acid in the first tube, using a second portion of 5 ml hexane (5.2.4).
- Combine the hexane phases in the second glass tube.
- Add 1 ml concentrated sulfuric acid (5.2.5) to the combined hexane phases. Place the tubes during 30 minutes in a water bath at 60 °C, mix carefully every 10 minutes.
- Leave the samples to cool to room temperature and centrifuge for 5 minutes at 2000 rpm.
- Transfer the hexane to a 50 ml PP screwcap centrifuge tube (Greiner) (5.2.17).
- Repeat the extraction of the suphuric acid in the second glass tube, using a portion of 5 ml hexane (5.2.4).
- Centrifuge for 5 minutes at 2000 rpm.
- Transfer and combine the hexane in the 50 ml PP screwcap centrifuge tube (Greiner).
- Add 0.2 ml 10 M Sodium hydroxide (5.2.7.1) and 5 ml deionized water (5.2.6) to the combined hexane in the Greiner tube.
- Shake during 1 minute and centrifuge for 5 minutes at 3500 rpm.
- Transfer the hexane to a second 50 ml PP screwcap centrifuge tube (Greiner) (5.2.17).
- Add 0.1 ml 10 M Sodium hydroxide (5.2.7.1) and 5 mL deionized water (5.2.6) to the hexane in the second Greiner tube.
- Shake during 1 minute and centrifuge for 5 minutes at 3500 rpm.
- Discard the hexane phase from the second Greiner tube.
- Combine the water phase from both Greiner tubes, in the second Greiner tube.
- Add 10 ml Acetonitrile (5.2.1) to the combined water phases and adjust the pH to <3 by adding 0.2 ml concentrated sulfuric acid (5.2.5) (check with pH paper).
- Mix the tube, add the pre-weight salts [4 gram Magnesium Sulphate (5.2.8) and 1 gram Sodium chloride (5.2.9)], shake immediately for 1 minute.

- Centrifuge for 5 minutes at 3500 rpm.
- Transfer 1 ml acetonitrile phase in a LC-MS vial (5.2.21).

9.3 LC-MSMS

9.3.1 Preparation of the system

Equilibrate the LC system under the recommended operating conditions (6.4).

Tune and calibrate the MS.

9.3.2 Checking instrument settings

Inject calibration level 5 (5.2.16) and check peak shape and retention time.

9.3.3 Determination

Inject a sufficient aliquot (e.g. 20 µL) of all the calibration standard solutions (5.2.16, 1 to 7) and an equal volume of the sample extracts.

Identify the PCP peak on basis of retention time and ion ratio.

Determine the amount of PCP by comparing the size of the sample peaks with those of the known amount in the calibration standard solutions (5.2.16, 1-7). Calibration is based on internal standard principle.

10 Calculation and expression of results

10.1 General

Calculations are performed using data acquisition software. Settings as below:

Before processing all data, the retention time of PCP is checked and, if necessary, modified in the processing method.

Use a seven-point internal standard calibration, force through zero (if no signal from blank sample is present), and calculate the correlation coefficient r^2.

After processing of the data, every result is manually checked for correct integration.

10.2 Calibration criteria

Criteria for Correlation coefficient: >0,995.

The results should fit within the calibration curve. When a result exceeds the thresholds of the calibration curve the sample should be diluted and reanalysed until it fits within the calibration curve.

10.3 Identification and confirmation

PCP is identified on retention time and mass ratio of the fragments. The relative intensities of the detected ions, expressed as a percentage of the intensity of the most intense ion, shall correspond to those of the calibration standard, either from calibration standard solutions or from spiked samples, at comparable concentrations, measured under the same conditions within the following tolerances:

Table 5. Relative intensities

Relative intensities (%of base peak)	Tolerances
> 50%	± 10%
> 20% to 50%	± 15%
> 10% to 20%	± 20%
<10%	± 50%

The ratio of the chromatographic retention time of PCP to that of the internal standard, the relative retention time, shall correspond to that of the calibration solution at a tolerance of ± 0.25%.

10.4 Calculation

For all calibration levels the relative response factor (RRF) is calculated.

$$RRF_{(n)} = \frac{A_x x Q_{is}}{Q_x x A_{is}}$$

where

A_x is the area of the Quantifier Ion of compound of interest;

A_{is} is the area of the Quantifier Ion of used internal standard;

Q_{is} is the Amount of internal standard ng/ml;

Q_x is the Amount of component of interest ng/ml.

Consequently the averaged relative response factor is calculated:

$$\overline{RRF_{(n)}} = \frac{1}{6} x \sum_{i=2}^{7} RRF_i(n)$$

where

n is the Compound of interest;

I is the calibration level (2-7) (level 1 is blank).

The concentration of the compound of interest is calculated by:

$$C_x = \frac{A_x x Q_{is} x F}{A_{is} x W x \overline{RRF_{(n)}}}$$

where

C_x is the Concentration component of interest in ng/g;

A_x is the area of the Quantifier Ion of compound of interest;

A_{is} is the area of the Quantifier Ion of used internal standard;

Q_{is} is the Amount of internal standard ng/ml;

F is the dilution factor (i.e., 10);

W is the Weight of the sample amount in g.

10.5 Recovery

The recovery of the used internal standard is calculated by:

$$percentage\ \ recovery\ \ (\%) = \frac{A_{sa}}{\overline{A_{st}}} x100$$

where

A_{sa} is the area of internal standard in the sample;

A_{st} is the average area of the internal standard in the Calibration level 1-7.

11 Precision

11.1 Collaborative test

Details of a collaborative trial on the precision of the method are shown in [4]. The values derived from this collaborative trial may not be applicable to concentration ranges and/or matrices other than those stated.

11.2 Repeatability limit

The absolute difference between two single test results found on identical test materials by one operator using the same apparatus within the shortest feasible time interval will exceed the repeatability limit r in not more than 5% of the cases. These limits can be found in Annex A.

11.3 Reproducibility limit

The absolute difference between two single test results on identical test materials reported by two laboratories will exceed the reproducibility limit R in not more than 5% of the cases. These limits can be found in Annex A.

12 Test report

1 information necessary for the identification of the sample;

2 a reference to this European Standard;

3 the date of sample receipt;

4 the test results and the units in which they have been expressed; where necessary the recoveries shall be stated along with the test results and whether the test results were corrected with those recoveries.

Bibliography

[1] ISO 6497:2002, *Animal feeding stuffs – Sampling*

[2] ISO 6498, *Animal feeding stuffs – Preparation of test samples*

[3] AOAC INTERNATIONAL. Appendix D: Guidelines for Collaborative Study Procedures To Validate Characteristics of a Method of Analysis. AOAC Official Methods of Analysis, 2005

[4] Gebbink et al., RIKILT – Report on the method validation study to standardize a method for the determination of pentachlorophenol (PCP) in feed materials and compound feed by LC-MS/MS.

[5] ISO 5725-2

Annex 5 Reported concentrations and Mandel outliers

Labcode	Feed 1		Feed 2		Guar gum		FAD 1		FAD 2	
µg/kg	A	B	C	D	E	F	G	H	I	J
PT084	9	8	14	14	17	19	14	11	22	22
PT092	9.56	9.29	14.07	13.19	15.2	16.72	5.76	7.46	17.88	17.3
PT141	7.75	7.5	12	12.5	12.5	13.25	14.5	14.25	28.75	31.25
PT142	8.35	8.03	13.45	13.61	16.8	15.33	11.04	11.66	28.54	27.47
PT143	22.1	19.9	17.5	18.1	15.1	13.9	12	10.6	20.5	19.6
PT144	5		2	1		3	9	11	19	8
PT145					13.3	14.4				
PT146	7.662	7.402	8.094	8.198	14.916	14.991	12.49	14.452	22.878	18.153
PT147					13.98	15.47			21.18	20.81
PT151	7.7	9	9.5	8.3			11	11	21.3	21.2
PT153	6.72	6.4	6.96	6.4	9.08	8.88	21.3	21.8	20.7	21.9
PT154	6	6	8	7	14	14	45	33	43	57
PT155	13.4	12	39.5	43.8	15.6	14.8		12.6		18.1
PT157	4.57	3.24	3.69	3.45	8.44	6.69	4.78	3.181	4.66	7.288
PT158	21.28	35.79	21.23	34.58	13.31	14.68	84.07	60.13	39.97	31.44
PT159	10.5	11	17.8	12.8	13.8	16.4	15.1	14.1	30.4	27.4

After removal of single datapoints (in orange) and Mandel statistics (in red)

Labcode	Feed 1		Feed 2		Guar gum		FAD 1		FAD 2	
µg/kg	A	B	C	D	E	F	G	H	I	J
PT084	9	8	14	14	17	19	14	11	22	22
PT092	9.56	9.29	14.07	13.19	15.2	16.72	5.76	7.46	17.88	17.3
PT141	7.75	7.5	12	12.5	12.5	13.25	14.5	14.25	28.75	31.25
PT142	8.35	8.03	13.45	13.61	16.8	15.33	11.04	11.66	28.54	27.47
PT143	22.1	19.9	17.5	18.1	15.1	13.9	12	10.6	20.5	19.6
PT144	5		2	1		3	9	11	19	8
PT145					13.3	14.4				
PT146	7.662	7.402	8.094	8.198	14.916	14.991	12.49	14.452	22.878	18.153
PT147					13.98	15.47			21.18	20.81
PT151	7.7	9	9.5	8.3			11	11	21.3	21.2
PT153	6.72	6.4	6.96	6.4	9.08	8.88	21.3	21.8	20.7	21.9
PT154	6	6	8	7	14	14	45	33	43	57
PT155	13.4	12	39.5	43.8	15.6	14.8		12.6		18.1
PT157	4.57	3.24	3.69	3.45	8.44	6.69	4.78	3.181	4.66	7.288
PT158	21.28	35.79	21.23	34.58	13.31	14.68	84.07	60.13	39.97	31.44
PT159	10.5	11	17.8	12.8	13.8	16.4	15.1	14.1	30.4	27.4

Final data set

Labcode	Feed 1		Feed 2		Guar gum		FAD 1		FAD 2	
µg/kg	A	B	C	D	E	F	G	H	I	J
PT084	9	8	14	14	17	19	14	11	22	22
PT092	9.56	9.29	14.07	13.19	15.2	16.72	5.76	7.46	17.88	17.3
PT141	7.75	7.5	12	12.5	12.5	13.25	14.5	14.25	28.75	31.25
PT142	8.35	8.03	13.45	13.61	16.8	15.33	11.04	11.66	28.54	27.47
PT143	22.1	19.9	17.5	18.1	15.1	13.9	12	10.6	20.5	19.6
PT144							9	11		
PT145					13.3	14.4				
PT146	7.662	7.402	8.094	8.198	14.916	14.991	12.49	14.452	22.878	18.153
PT147					13.98	15.47			21.18	20.81
PT151	7.7	9	9.5	8.3			11	11	21.3	21.2
PT153	6.72	6.4	6.96	6.4	9.08	8.88	21.3	21.8	20.7	21.9
PT154	6	6	8	7	14	14	45	33	43	57
PT155	13.4	12	39.5	43.8	15.6	14.8				
PT157	4.57	3.24	3.69	3.45	8.44	6.69	4.78	3.181		
PT158			21.23	34.58	13.31	14.68			39.97	31.44
PT159	10.5	11	17.8	12.8	13.8	16.4	15.1	14.1	30.4	27.4
spiked	8		13		15		10		20	
homogeneity test	9.1		13.5		14.8		11.1		22.1	

Annex 6 Mandel h and k graphs

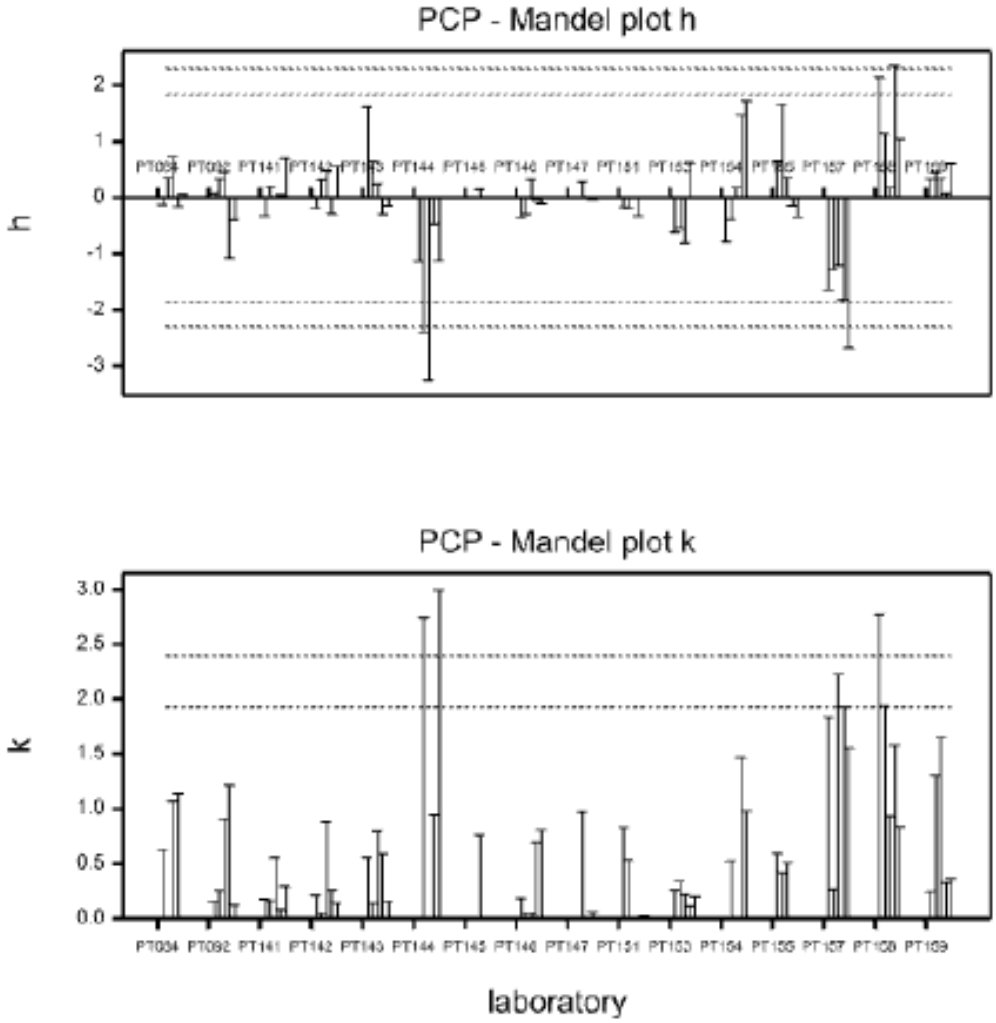

Figure 1. Mandel h and k statistics as five bars, representing the five sample types, in the order types (Feed 1, Feed 2, Guar, FAD 1, FAD 2). The dotted lines are the critical values at the 1% and 5% significance level

Annex 7 Modifications to method by participants

PT092 LCMS conditions were amended as agreed in email

PT142 Filtered extracted sample (1ml) over 0.2um nylon syringe filter.

PT143 Changed mobile phase gradient in order to separate the interference from the PCP peak.

PT145 Guar gum: Column temperature: 50 degrees Celcius

Injection volume: 5 µL

Gradient:

Time	Mobile A	Mobile B
0.00	60	40
0.10	60	40
3.00	0	100
5.00	0	100
5.50	60	40
7.00	60	40

PT151 A different sample amount was analysed for the guar gum samples. The participant analysed the whole vial containing approximately 3 gram guar gum instead of the prescribed 1.0 gram. As the exact sample weight was not recorded by the participant, a concentration could not be calculated.

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