Preamble

- **Protocols of validation:**

- **EN ISO 16140-1 and EN ISO 16140-2 (September 2016): Microbiology of the food chain — Method validation**
  Part 1: Vocabulary.
  Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method.

- **Requirements regarding comparison and interlaboratory studies for implementation of the standard EN ISO 16140-2 (version 6).**

- **Reference method:**

- **EN ISO 6579-1 (April 2017):** Microbiology of the food chain – Horizontal method for the detection, enumeration and serotyping of *Salmonella*- Part 1: Detection of *Salmonella* spp.

- **Application scope:**

- **All human food products** by a validation testing of a broad range of foods, including:
  - meat products,
  - dairy products,
  - seafood products,
  - egg products,
  - ready-to-eat and ready-to-reheat products,
- **Feed products,**
- **Environmental samples.**

- **Certification body:**

- **AFNOR Certification** (https://nf-validation.afnor.org/).

Definitions

- **Method comparison study**

The method comparison study is the part of the validation process that is performed in the organizing laboratory. It consists of three parts namely the following:
- A comparative study of the results of the reference method to the results of the alternative method in (naturally and/or artificially) contaminated samples (so-called sensitivity study);
- A comparative study to determine the relative level of detection (RLOD) in artificially contaminated samples (so-called RLOD study);
- An inclusivity/exclusivity study of the alternative method.

- **Sensitivity study**

The sensitivity study aims to determine the difference in sensitivity between the reference and the alternative method.
The sensitivity is the ability of the reference method or alternative method to detect the analyte.

- **Relative level of detection study**

A comparative study is conducted to evaluate the level of detection (LOD) of the alternative method against the reference method. The evaluation is based on the calculation of the relative level of detection (RLOD).
The level of detection at 50% (LOD_{50}) is the measured analyte concentration, obtained by a given measurement procedure, for which the probability of detection is 50%.
The relative level of detection level of detection at P = 0,50 (LOD_{50}) of the alternative method divided by the level of detection at P = 0,50 (LOD_{50}) of the reference method.

- **Inclusivity and exclusivity study**

The inclusivity study is a study involving pure target strains to be detected or enumerated by the alternative method.
The exclusivity study is a study involving pure non-target strains, which can be potentially cross-reactive, but are not expected to be detected or enumerated by the alternative method.

- **Interlaboratory study**

The interlaboratory study is a study performed by multiple laboratories testing identical samples at the same time, the results of which are used to estimate alternative-method performance parameters.
The aim of the interlaboratory study is to determine the difference in sensitivity between the reference and the alternative method when tested by different collaborators using identical samples (reproducibility conditions).

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Appendices

1. Introduction

The Simple Method for *Salmonella* method (SMS) is validated by AFNOR Certification under the mark NF VALIDATION with the certification number AES 10/04-05/04 according to the standard EN ISO 16140/A1:2011. The method is intended for all human food products, feed products and environmental samples (except primary production samples) since its initial validation.

Table 1 summarizes the different steps of the validation that occurred since the initial validation.

Table 1: Steps of the validation AFNOR certification

Study	Date	Standard	Expert Laboratory	Observation
Initial validation	May 2004	ISO 16140:2003 ISO 6579:2002	Institut Scientifique d'Hygiène et d'Analyse	/
Extension	July 2007	ISO 16140:2003 ISO 6579:2002	Institut Scientifique d'Hygiène et d'Analyse	Addition of a confirmation test
First renewal	March 2008	ISO 16140:2003 ISO 6579:2002	Institut Scientifique d'Hygiène et d'Analyse	No additional tests
Second renewal	March 2012	ISO 16140/A1:2011 ISO 6579:2002	Institut Scientifique d'Hygiène et d'Analyse	No additional tests
Third renewal	May 2016	ISO 16140/A1:2011 ISO 6579:2002	Institut Scientifique d'Hygiène et d'Analyse	Removal of samples contaminated at a level >30 CFU/25 g Additional tests to replace these samples. Determination of the RLOD of the feed category
Fourth renewal study	November 2019	ISO 16140-2: 2016 ISO 6579-1:2017	Microsept	Additional tests to fulfill the requirements of the revised standard

The present document introduces all the validation studies results for the NF VALIDATION certification of the SMS method.

A part of the results set out in this report were produced during validation tests carried out by Institut Scientifique d'Hygiène et d'Analyse as part of NF Validation, in accordance with prevailing requirements.

The remaining part of the results is constituted by the analyses performed by the Laboratory Microsept as part of the requirements of the updated validation standard.

2. Protocols of the methods

2.1. Alternative method

2.1.1. Principle of the method

SMS principle lies on the motility of *Salmonella* and on their ability to decarboxylate L-Lysine. On SMS, *Salmonella* produce a red and opaque halo of migration around the original point of inoculation. The medium selective agents and an incubation at 41°C give to SMS a strong selectivity. The gelling base of the medium was especially optimized to authorize easy transport and handling of ready poured medium while ensuring an optimal migration of the motile *Salmonella* (deposited patent).

2.1.2. Protocol of the method

The protocol is as follows:

- enrichment in buffered peptone water, incubated for 16 to 20 hours at 37°C ± 1°C,
- inoculation of 3 spots of 0.1 ml at 5 mm of the edge of the Petri dish on a SMS medium, incubated for 14 to 25 hours at 41°C ± 1°C.

The workflow of the method is set out in Appendix A.

2.1.3. Restrictions

Non motile *Salmonella* cannot be detected with SMS method.

2.2. Reference method

The standard EN ISO 6579:2002 was used for the initial validation study and for the following renewal studies.

This standard was revised in 2017 and the amendments introduced were considered minor. It's consequently the EN ISO 6579-1 (April 2017) standard: *Horizontal method for the detection, enumeration and serotyping of Salmonella - Part 1: Detection of Salmonella spp* that was used as a reference method during the tests performed for the present renewal study.

The workflow of the reference method is presented in Appendix B.
Further explanations regarding this update are provided in paragraph 9.2.

The workflow of the reference method is presented in Appendix B.

2.3. Study design

As there is a shared enrichment step for both the alternative and the reference methods, the same test portion is used for both methods. The study will thus provide paired data and the expression “paired study” is used to describe the study design.

3. Methods comparison study

The study was conducted on a variety of samples and strains representative of food products. This is not an exhaustive list of the various matrices included in the application scope. For any remark on the alternative method, you can contact AFNOR Certification by connecting to the Internet page ***http://nf-validation.afnor.org/contact-2/***.

3.1. Sensitivity study

The purpose of this study is to compare the two methods – the reference method EN ISO 6579-1 and the SMS method – on samples contaminated or not contaminated with S*almonella.*

3.1.1. Protocols applied during the validation study

- **Incubation times:**

The minimum incubation times were tested, namely 16 hours for the enrichment in Buffered Peptone Water and 14 hours and 24 hours for the SMS plates.

- **Confirmations:**

Presumptive positive results were confirmed by the realization of two protocols: a five hours protocol using a tube of BHI and a latex test and a 24 hours protocol using the SALSA medium and a latex test.

3.1.2. Number and nature of the samples

The sensitivity study for all categories concerned 526 samples:

- 315 samples analyzed during the initial validation study,
- 211 samples analyzed during this fourth renewal study.

Samples analyzed by category and type are presented in table 2.

Table 2: Distribution of the samples per category and type (: by any method)*

Category		Type	Positive results*	Negative results	Total
Meat products ①	a	Raw meats	16	17	33
	b	Raw poultry	11	9	20
	c	Delicatessen, RTE	10	12	22
		Total	**37**	**38**	**75**
Dairy products ②	a	Cow's milk cheese	10	27	37
	b	Goat and ewe milk cheese	10	10	20
	c	Other dairy products	10	10	20
		Total	**30**	**47**	**77**
Seafood products ③	a	Fish	9	24	33
	b	Molluscs/Shelfishs	11	10	21
	c	Other seafood products	10	10	20
		Total	**30**	**44**	**74**
Egg products ④	a	Liquid eggs	11	9	20
	b	Egg powders	10	10	20
	c	Eg products	10	24	34
		Total	**31**	**43**	**74**
Ready-to-eat and ready-to-reheat products ⑤	a	Ready-to-eat products	10	11	21
	b	Ready-to-reheat products	10	11	21
	c	Smoked, marinated products	10	12	22
		Total	**30**	**34**	**64**
Feed products ⑥	a	Wet pet foods	10	12	22
	b	Dry pet foods	10	16	26
	c	Cattle feed	10	12	22
		Total	**30**	**40**	**70**
Environmental samples ⑦	a	Process waters	11	10	21
	b	Surfaces	16	31	47
	c	Wastes	14	10	24
		Total	**41**	**51**	**92**
All categories		**Total**	**229**	**297**	**526**

3.1.3. Artificial contamination

Artificial contamination was carried out using stressed strains in accordance with the requirements of the validation standard and the AFNOR Validation Technical Board (see Appendix C).
Table 3 gives the distribution of the positive samples per level of contamination.

Table 3: distribution of the positive samples per level (cl: contamination level)

Positive samples	Naturally contaminated samples	Artificially contaminated samples						Total
		Spiking			Seeding			
		cl ≤ 5	5 < cl ≤ 10	10 < cl ≤ 30	cl ≤ 3	3 < cl ≤ 10	cl > 10	
229	51	44	2	13	118	1	0	229
%	22,3%	19,2%	0,9%	5,7%	51,5%	0,4%	0%	100%

229 samples gave a positive result by at least one of the methods and 22,3% of them were naturally contaminated.

One hundred and three results obtained during the initial validation with samples contaminated at levels greater than 10 CFU per test portion and 13 results, also obtained during the initial validation study, with unknown contamination levels were not included in the statistical interpretation to fulfill the requirements of the Technical Board (last table of the sensitivity appendices). They concern:

- 21 dairy products in positive agreement,
- 25 seafood products in positive agreement,
- 17 egg products in positive agreement,
- 26 environment samples in positive agreement,
- 27 feed products in positive agreement.

3.1.4. Results

Raw data are shown in appendix D.
Table 4 shows the results of the sensitivity study for all categories.

Table 4: results of the sensitivity study for both methods (R+/-: reference method positive or negative, A+/-: alternative method positive or negative, PA: positive agreement, NA: negative agreement, ND: negative deviation, PD: positive deviation, PP: presumptive positive before confirmation)

Category	Response	R+	R-
Meat products ❶	A+	PA = 36	PD = 0
	A-	ND = 1 incl. 0 PPND	NA = 38 incl. 0 PPNA
Dairy products ❷	A+	PA = 25	PD = 3
	A-	ND = 2 incl. 0 PPND	NA = 47 incl. 0 PPNA
Seafood products ❸	A+	PA = 29	PD = 1
	A-	ND = 0 incl. 0 PPND	NA = 44 incl. 0 PPNA
Egg products ❹	A+	PA = 31	PD = 0
	A-	ND = 0 incl. 0 PPND	NA = 43 incl. 0 PPNA
Ready-to-eat and ready-to-reheat products ❺	A+	PA = 30	PD = 0
	A-	ND = 0 incl. 0 PPND	NA = 34 incl. 0 PPNA
Feed products ❻	A+	PA = 30	PD = 0
	A-	ND = 0 incl. 0 PPND	NA = 40 incl. 0 PPNA
Environmental samples ❼	A+	PA = 40	PD = 1
	A-	ND = 0 incl. 0 PPND	NA = 51 incl. 0 PPNA
All categories	A+	**PA = 221**	**PD = 5**
	A-	**ND = 3 incl. 0 PPND**	**NA = 297 incl. 0 PPNA**

3.1.5. Calculation of relative trueness (RT), sensitivity (SE) and false positive ratio (PFR)

The set of results obtained were used to calculate the relative trueness, the sensitivity and the false positive ratio for each of the categories and for all the categories, according to the formulas set out in the EN ISO 16140-2:2016 standard (table 5).

Table 5: values in % of sensitivity for the two methods, relative trueness and false positive ratio for the alternative method (SE_{alt}: sensitivity for the alternative method, SE_{ref}: sensitivity for the reference method, RT: relative trueness, FPR: false positive ratio for the alternative method)

Categories	Type	PA	NA	ND	PD	N	PPND	PPNA	SEalt	SEref	RT	FPR
Meats products ①	a	16	17	0	0	33	0	0	100,0%	100,0%	100,0%	0,0%
	b	11	9	0	0	20	0	0	100,0%	100,0%	100,0%	0,0%
	c	9	12	1	0	22	0	0	90,0%	100,0%	95,5%	0,0%
	Total	36	38	1	0	75	0	0	97,3%	100,0%	98,7%	0,0%
Dairy products ②	a	10	27	0	0	37	0	0	100,0%	100,0%	100,0%	0,0%
	b	9	10	0	1	20	0	0	100,0%	90,0%	95,0%	0,0%
	c	6	10	2	2	20	0	0	80,0%	80,0%	80,0%	0,0%
	Total	25	47	2	3	77	0	0	93,3%	90,0%	93,5%	0,0%
Seafood products ③	a	9	24	0	0	33	0	0	100,0%	100,0%	100,0%	0,0%
	b	10	10	0	1	21	0	0	100,0%	90,9%	95,2%	0,0%
	c	10	10	0	0	20	0	0	100,0%	100,0%	100,0%	0,0%
	Total	29	44	0	1	74	0	0	100,0%	96,7%	98,6%	0,0%
Egg products ④	a	11	9	0	0	20	0	0	100,0%	100,0%	100,0%	0,0%
	b	10	10	0	0	20	0	0	100,0%	100,0%	100,0%	0,0%
	c	10	24	0	0	34	0	0	100,0%	100,0%	100,0%	0,0%
	Total	31	43	0	0	74	0	0	100,0%	100,0%	100,0%	0,0%
Ready-to-eat and reheat products ⑤	a	10	11	0	0	21	0	0	100,0%	100,0%	100,0%	0,0%
	b	10	11	0	0	21	0	0	100,0%	100,0%	100,0%	0,0%
	c	10	12	0	0	22	0	0	100,0%	100,0%	100,0%	0,0%
	Total	30	34	0	0	64	0	0	100,0%	100,0%	100,0%	0,0%
Feed products ⑥	a	10	12	0	0	22	0	0	100,0%	100,0%	100,0%	0,0%
	b	10	16	0	0	26	0	0	100,0%	100,0%	100,0%	0,0%
	c	10	12	0	0	22	0	0	100,0%	100,0%	100,0%	0,0%
	Total	30	40	0	0	70	0	0	100,0%	100,0%	100,0%	0,0%
Environmental samples ⑦	a	11	10	0	0	21	0	0	100,0%	100,0%	100,0%	0,0%
	b	16	31	0	0	47	0	0	100,0%	100,0%	100,0%	0,0%
	c	13	10	0	1	24	0	0	100,0%	92,9%	95,8%	0,0%
	Total	40	51	0	1	92	0	0	100,0%	97,6%	98,9%	0,0%
All categories	**Total**	**221**	**297**	**3**	**5**	**526**	**0**	**0**	98,7%	97,8%	98,5%	0,0%

The results for all categories are summarized in the table 6 below.

Table 6: summary of the results for all categories

Parameter	Formula EN ISO 16140-2 :2016	Results for all categories
Sensitivity of the alternative method (SE_{alt})	$SE_{alt} = \frac{(PA + PD)}{(PA + ND + PD)} \times 100\ \%$	98,7 %
Sensitivity of the reference method (SE_{ref})	$SE_{ref} = \frac{(PA + ND)}{(PA + ND + PD)} \times 100\ \%$	97,8 %
Relative trueness (RT)	$RT = \frac{(PA + NA)}{N} \times 100\ \%$	98,5 %
False positive ratio (FPR)	$FPR = \frac{FP}{NA} \times 100\ \%$	0 %

3.1.6. Analysis of discordant results

Discordant results are examined according to the standard ISO 16140-2: 2016.

- **Positive deviations**:

Five samples gave positive deviations:

Three samples gave a positive deviation in the category “Dairy products”: 2 samples of raw milk (L16 and L18), naturally contaminated and a raw goat milk cheese (1758419), artificially contaminated. The level of *Salmonella* for sample number 1758419 is equal to 1,6 CFU per test portion. With the SMS method the confirmation of *Salmonella* was only possible after 24 hours of incubation. After 14 hours of incubation the migration area was too weak.

One sample gave a positive deviation in the category “Seafood”: a mussel sample (M2), naturally contaminated.

One sample gave a positive deviation in the category “Environment”: an egg product residue (1778803), artificially contaminated. With the reference method, a high proportion of annex flora was found on XLD and ASAP media.

- **Negative deviations:**

Three samples gave negative deviations: a sample of raw ravioli (C26), naturally contaminated, a sample of fermented milk (SMS 23), artificially contaminated and a sample of raw cow’s milk (SMS 42), artificially contaminated.

For fermented milk, the inoculation of *Salmonella* is low at a level of 0,4 CFU. Three plates of SMS media were inoculated and were all found negative. With the reference method, no colony was found on XLD and ASAP media from the RVS broth and only four colonies were detected on XLD and ASAP media from MKTTn broth.

For raw cow’s milk, the inoculation level is equal to 1. Five plates of SMS media were inoculated and were all found negative.

3.1.7. Calculation and interpretation of data

Table 7 shows the difference between negative deviations and positive deviations and the acceptability limits.

Table 7: acceptability limits (AL)

Category	Type	ND	PD	(ND-PD)	(AL)	(ND+PD)	(AL)	Observation
Meat products ①	a	0	0	/	/	/	/	(ND-PD) ≤ AL:
	b	0	0					
	c	1	0					
	Total	1	0	1	3	1	6	
Dairy products ②	a	0	0	/	/	/	/	
	b	0	1					
	c	2	2					
	Total	2	3	-1	3	5	6	
Seafood products ③	a	0	0	/	/	/	/	
	b	0	1					
	c	0	0					
	Total	0	1	-1	3	1	6	
Egg products ④	a	0	0	/	/	/	/	
	b	0	0					
	c	0	0					
	Total	0	0	0	3	0	6	
Ready-to-eat and ready-to-reheat products ⑤	a	0	0	/	/	/	/	
	b	0	0					
	c	0	0					
	Total	0	0	0	3	0	6	
Feed products ⑥	a	0	0	/	/	/	/	
	b	0	0					
	c	0	0					
	Total	0	0	0	3	0	6	
Environmental samples ⑦	a	0	0	/	/	/	/	
	b	0	0					
	c	0	1					
	Total	0	1	-1	3	1	6	
All categories	***Total***	**3**	**5**	**-2**	**6**	**9**	**18**	

The observed values (ND – PD) and (ND + PD) are below the acceptability limit for each category and for all categories. The alternative method produces results comparable to the reference method.

3.1.8. Confirmation

All the SMS plates with a positive profile were confirmed by the two confirmation protocols: the five hours protocol using a culture in BHI followed by a latex test and the 24 hours protocol using the SALSA medium and latex test.

3.1.9. Conclusion of the sensitivity study

The statistical tests of the EN ISO 16140-2:2016 standard conclude that the alternative method produces comparable results to the reference method.

3.2. Relative detection level study

3.2.1. Matrices used

Various "food matrix-strain" pairs were studied in parallel using the reference method and the alternative method, for the studied categories (cf. table 8).

Table 8: couples matrix-strain for each category

Category	Couple matrix strain	Origin of the strain	Step of the validation
❶	Minced meat / *Salmonella* Typhimurium	Meat product	Initial validation study according to ISO 16140:2003 standard
❷	Raw milk / *Salmonella* Dublin	Dairy product	
❸	Saithe fillet / *Salmonella* Virchow	Seafood product	
❹	Egg / *Salmonella* Enteritidis	Egg product	
❺	Mixed vegetables / *Salmonella* Infantis DGR133	Fresh leaves salad	4th renewal study acc. to ISO 16140-2:2016 standard
❻	Dog food / *Salmonella* Senftenberg	Soymeal	3rd renewal study acc.ISO 16140¬2:2016 standard
❼	Water process / *Salmonella* Typhimurium	Environmental sample	Initial validation study according to ISO 16140:2003 standard

The total flora of the matrix was determined and is set out in the results tables in appendix E.

3.2.2. Contamination protocol

3.2.2.1. Initial validation study

Four levels of contamination were tested including the negative control.

Six replicates for each level of contamination were inoculated and analyzed by the reference method and the alternative method.
As the two methods have a common step, 6 test portions of 25 g were prepared for each level of contamination and individually inoculated with a calibrated bacterial suspension.

Bacterial suspensions of about 1 cell per mL were prepared. From these initial suspensions, volumes of 0.9 mL, 0.3 mL and 0.1 mL were used to spike 25 g of sample respectively for the 3 first levels. For all the levels of contamination, homogeneity of the inoculums was checked by enumeration on 30 TSA Petri dishes. A level “0” without contamination was also realized.

3.2.2.1. Third and fourth renewal studies

Three levels of contamination were tested including the negative control.

The negative control level shall not produce positive results. Five replicates are tested for this level.

The low level shall be the theoretical detection level, it has been contaminated at 0.7 - 1 CFU per test portion to obtain fractional recovery results. Twenty replicates are tested for this level.

The higher level shall be just above the theoretical detection level, it has been contaminated at 2 - 3 CFU per test portion. Five replicates are tested for this level.

The matrix was contaminated using the seeding protocol. Bulk contaminations were performed on the matrix for the different levels of contamination, then the matrix was stored at 5±3°C for two days before analysis.

3.2.3. Results

The detailed results tables are set out in Appendix E.
The RLOD is defined as the ratio of the LODs of the alternative method and the reference method: RLOD= LOD_{alt} / LOD_{ref}.

The RLODs calculations were performed according to the standard ISO 16140-2: 2016 using the Excel spreadsheet available for download at http://standards.iso.org/iso/16140, with unknown concentrations. Values of the RLODs are set out in table 9.

Table 9: RLODs values for all categories (RLOD: the estimated relative level of detection value, RLODU: the upper limit of the 95% ,584confidence interval for RLOD, RLODL: the lower limit of the 95% confidence interval for RLOD, b=ln(RLOD): logarithm of the RLOD value, sd(b): standard deviation of b, z-Test statistic: absolute value of the test statistic of the z-Test with the null hypothesis H0: b=0, p-value: p-value of the z-Test)

Category	RLOD	RLODL	RLODU	b=ln(RLOD)	sd(b)	z-Test statistic	p-value	Accep-tability limit
❶	1,000	0,311	3,217	0,000	0,584	0,000	1,000	
❷	1,000	0,287	3,489	0,000	0,625	0,000	1,000	
❸	1,000	0,311	3,217	0,000	0,584	0,000	1,000	
❹	1,000	0,311	3,217	0,000	0,584	0,000	1,000	
❺	1,000	0,466	2,145	0,000	0,382	0,000	1,000	1,5
❻	1,000	0,403	2,480	0,000	0,454	0,000	1,000	
❼	1,000	0,311	3,217	0,000	0,584	0,000	1,000	
Combined	**1,000**	**0,706**	**1,417**	**0,000**	**0,174**	**0,000**	**1,000**	

The LOD_{50} calculations according to Wilrich & Wilrich POD-LOD calculation program - version 9, are given in table 10.

Table 10: LOD50% for the alternative and reference method

Matrix	Strain	LOD50% (CFU/25g) alternative method	LOD50% (CFU/25g) Reference method
Minced meat	*Salmonella* Typhimurium	0,394	0,394
Raw milk	*Salmonella* Dublin	0,451	0,451
Saithe fillet	*Salmonella* Virchow	0,238	0,238
Egg	*Salmonella* Enteritidis	0,315	0,315
Mixed vegetables	*Salmonella* Infantis	0,497	0,497
Dog food	*Salmonella* Senftenberg	0,943	0,943
Water process	*Salmonella* Typhimurium	0,394	0,394
Combined results		**0,475**	**0,475**

3.2.4. Interpretation and conclusion

The RLODs values are below the acceptability limit set at 1,5 meaning that, as stated in ISO 16140-2:2016, the maximum increase in LOD of the alternative versus the reference method is not considered as relevant in consideration of the fitness for purpose of the method.

In conclusion, alternative and reference methods show similar LODs values for the detection of *Salmonella* spp in the categories tested.

3.3. Inclusivity and exclusivity study

The inclusivity and exclusivity of the method are defined by analyzing, respectively, 105 positive strains and 36 negative strains.

The inclusivity and exclusivity were tested in three steps:

- Initial validation study (2004): 54 target strains and 30 non-target strains,
- Second renewal study (2012): 23 target strains and 6 non-target strains,
- Fourth renewal study (2019): 28 target strains.

3.3.1. Test protocols

- **Protocol for inclusivity**

For each of the *Salmonella* strains tested, a culture in brain hearth infusion broth was performed for 24 hours at 37°C.
The buffered peptone water was inoculated between 10 and 100 cells per 225 ml, then the complete protocol of the method was applied.

- **Protocol for exclusivity**

The non-target strains were cultured in brain hearth infusion broth for 24 hours at 37°C, inoculated in 225 ml of buffered peptone water in order to obtain levels of around 10^5 cells per ml, then the complete protocol of the method was applied.

3.3.2. Results

The results are set out in Appendix F.

- **Inclusivity**

Among the 105 target strains,

- an arc of migration and a red coloration of the medium was observed for 95 strains,
- an arc of migration associated with a low red coloration or an absence of coloration was observed for the 5 strains of *S.* Paratyphi A (weak or absence of lysine decarboxylation activity),
- 2 strains of *S.* Gallinarum and the non-motile variant of *Salmonella* Typhimurium (non-motile *Salmonella* strains) gave negative results as expected (absence of migration),
- 1 *S.* Infantis strain and 1 *S.* Paratyphi C strain gave negative results (however 3 other strains of *Salmonella* Infantis and another strain of Salmonella Paratyphi C gave positive results),
- 1 *S*. Abortusequi strain gave weak of lysine decarboxylation activity and a migration arc of less than 2 cm. With the addition of skimmed milk powder, the strain gave a positive result despite an low decolouration of the agar media,
- 1 *S*. Lille strain and 1 *S*. Meleagridis strain gave a migration arc of less than 2 cm. With the addition of skimmed milk powder, the strains gave positive results,
- *1 S*. *houtenae* strain and 1 *S. bongor*i strain were characteristic on SMS but the agglutination with the latex test was respectively extremely fine and irregular.

- **Exclusivity**

No cross-reactions were observed with the 36 non-targets strains.

3.3.3. Conclusion

The inclusivity and the exclusivity of the alternative method are satisfactory.

3.4. Extension study

An extension study was performed in 2007 (documents in Appendix H).

3.4.1. Object of the extension

This study aimed to add an option for additional confirmation.

Tests were performed using pure cultures inoculated on the SMS media and showing a characteristic profile: 150 strains of *Salmonella* serotypes from various origins were tested and 105 non-target strains (their choice being guided by the genetic similarity with *Salmonella* spp.)

3.4.2. Protocols

Two protocols were tested:

- **Protocol 5 hours**

By streaking from a presumptively positive SMS Petri dish in brain heart infusion broth (BHI) and incubated for 5±1 h at 37±1°C followed by an agglutination test (antigen-antibody) latex.

- **Protocol 24 hours**

By streaking from a presumptively positive SMS Petri dish on SALSA medium and incubated for 21±3 h at 37±1°C followed by an agglutination test (antigen-antibody) latex.

SALSA agar medium is composed of 2 specific and selective media (XLD and ASAP) for *Salmonella* spp.

The medium SALSA is arranged in dual Petri dish:

- ASAP (white): its mode of action is based on the detection of enzyme activity (C8-esterase) which specifically cleaves a chromogenic substrate and colors the colonies of *Salmonella* in pink.
- XLD (red): its mode of action is based on the decarboxylation of L-lysine and / or production of hydrogen sulfide (H_2S) giving red colonies with or without black centers.

Agglutination test (*Salmonella* latex test reference MGNF42) uses latex particles sensitized with rabbit antibodies which agglutinates to *Salmonella* spp antigens forming aggregates clearly visible.

3.4.3. Results

3.4.3.1. Results for target strains

The results obtained for 144 strains are consistent with those expected. Strains with a positive profile on SMS agar form characteristics colonies on ASAP medium and / or XLD medium and show a positive reaction in the agglutination assay.

Seven of 150 strains gave a result different than expected as reported in the table below

Strain	Ref.	Profile on SMS	Latex test		SALSA			
					XLD		ASAP	
			BHI 5h	BHI 24h	CC	Latex	CC	Latex
S. arizonae	P64	-	/	/	/	/	/	/
S. Braenderup	P58	-	/	/	/	/	/	/
S. Cerro	P24	+	-	-	yes	auto	yes	auto
S. diarizonae	P65	-	/	/	/	/	/	/
S. salamae	P59	+	+	+	yes	auto	yes	auto
S. Urbana	P24	+	+	+	yes	auto	yes	auto
S. Paratyphi C	R106	-	/	/	no	+	yes	+

- 3 strains [*S. arizonae* (P64), *S.* Braenderup (P58) and *S. diarizonae* (P65)] have a negative profile on the SMS medium. The results obtained from the TSA are consistent with those expected (typical colonies on XLD and ASAP and positive reaction in the agglutination assay). Two *S.* arizonae , 1 *S.* Braenderup and 2 *S. diarizonae* strains were positive on SMS and with the confirmation tests.
- -3 strains are self-agglutinating [*S.* Cerro (P24), *S. salamae* (P62) and *S. Urbana* (P54)]. The latex test, from colonies obtained on different agar media (ASAP, XLD, TSA), is unusable. The agglutination reaction is positive from the BHI broth (at 5 and 24 h of incubation) for P62 and P54 strains, while the result is negative for the strain P24. Additional testing (confirmatory tests according to standard NF EN ISO 6579) show that it is a strain of *Salmonella* spp.

- 1 strain of *Salmonella* Paratyphi C (R106) is negative on SMS medium. From the colonies obtained on the TSA, R106 does not form characteristic colonies on XLD and the agglutination reaction is positive for the strain regardless of the modality tested.

Remarks:

- 5 strains form typical colonies on the ASAP medium and atypical colonies on XLD medium [*S.* London (P17), *S.* Montevideo (P25), *S.* Regent (P53), *S.* Tennessee (P21) and *S.* Worthington (P18)].
- 1 strain gives characteristic colonies on XLD medium and atypical colonies on the ASAP medium [*S.* Dublin (S59)].
- 2 strains of *Salmonella* Paratyphi A (R105 and R107) are colorless on SMS medium, due to the absence of lysine decarboxylase activity in these strains.

At the request of the Technical Committee, additional tests were performed.

For 30 target strains (30 different serotypes), the SMS agar plates showing a positive profile were stored at 5±3°C for 48 hours. The analytical protocol (BHI, SALSA and agglutination test) was then applied.

The results obtained from an SMS agar positive after storage for 48 hours at 5±3°C were identical to results obtained with the general protocol.

3.4.3.2. Results for non-target strains

The results are consistent with those expected. All strains tested gave a negative profile on SMS agar. No strain gave a positive latex agglutination test, except for a strain of *Serratia marcescens* (W34).

For *Serratia marcescens* (W34), the reaction is obtained in the form of filamentous aggregates which can be confused with a normal agglutination reaction.

Remarks:

Some non-target strains formed characteristic colonies on XLD and ASAP media:

- 4 strains formed characteristic colonies on XLD medium [*Citrobacter freundii* (R35), *Citrobacter freundii* (W3), *Proteus mirabilis* (W29) and *Proteus mirabilis* (W30)],
- 2 strains formed characteristic colonies on the ASAP medium [*Enterobacter sakazakii* (I37) and *Pseudomonas fluorescens* (R4)].

However, the latex agglutination tests performed on typical colonies formed by these strains are negative.

3.5. Practicability

The practicability of the alternative method was informed according to the criteria defined by the Technical Committee.

1. Storage conditions, shelf-life and modalities of utilization after first use

SMS agar is available:

- In pre-poured plates: 20 x 90 mm plates,

- In pre-poured plates: 120 x 90 mm plates,
- In bottle ready to regenerate: 6 bottles of 200 ml.

The shelf-life of tests is indicated on the reagents.
Pre-poured plates and bottles should be stored between +2°C and +8°C.

2. Time-to-result

Negative results are obtained in two days.
Positive results are obtained in:

- Two days using the 5 hours protocol and three days using the 24h protocol,
- Four days using the tests of the reference method.

3. Common step with the reference method

The enrichment step is common between the alternative method and reference method.

3.6. Conclusion

The comparative study of the methods was performed according to the EN ISO 16140-2:2016 standard.

- **Sensitivity study**

The performance of the SMS method was compared to that of the EN ISO 6579-1:2017 reference method by analyzing 526 samples divided into seven product categories.
The observed values (ND – PD) and (ND + PD) were below or equal to the acceptability limit for each category and for all categories.
Statistically, the alternative method produces results comparable to that of the reference method.

- **Relative level of detection study**

The relative detection level of the SMS method and reference method was evaluated by artificially contaminating seven different products.
The relative level of detection of the alternative method is equal to 1 cell per test portion.
The SMS method and the reference method showed similar LODs values for the detection of *Salmonella* spp in the categories tested.

- **Inclusivity and exclusivity study**

The specificity of the method is satisfactory, all the tested serovars of Salmonella were detected by the alternative method at the incubation times specified in the protocol, except non-motile strains of *Salmonella*. No cross-reactions were observed among non-targeted tested strains that were unable to be confirmed (exclusivity).

4. Interlaboratory study

4.1. Study organization

- **Number of participating laboratories**: fourteen collaborators received samples.
- **Matrix used:** pasteurized milk was used as matrix for the interlaboratory study.
- **Strain used:** the strain used for contamination was a strain of *Salmonella* Enteritidis isolated from an egg product.
- **Number of samples per laboratory:** 24 samples per collaborator were prepared for the reference method and 24 samples for the alternative method, broken down into 3 levels, with 8 samples per level. One additional sample, not artificially contaminated, was provided to the collaborators for the enumeration of the microorganisms of the matrix.

4.2. Control of the experimental parameters

4.2.1. Contamination level

The contamination rates obtained in the matrix are set out in the table below:

Table 11: theoretical and actual contamination levels

Level	Samples	Theoretical target level (CFU / 25 ml)	Real level (CFU / 25 ml)
L_0: Level 0	2/9/14/17/21/22/23/24	0	0
L_1: Low level	3/4/10/11/12/13/19/20	3	3
L_2: High level	1/5/6/7/8/15/16/18	30	31

4.2.2. Stability of the samples

The strain stability in the pasteurized milk matrix was evaluated for 5 days at (4±2)°C. Two kinds of analyses were performed:

- Inoculation at 3 CFU/25 ml and detection by the alternative method and the reference method at D0, D1, D2 and D5
- Inoculation at $2{,}3.10^4$ CFU/ml in vials of 20 ml and enumeration on Hektoen agar media at D0, D1, D2 and D5

The results are summarized in table 12.

Table 12: stability of the samples

Day	Alternative method	Reference method	Enumeration (CFU/ml)
D0	Presence in 25 mL	Presence in 25 mL	900
D+1	Presence in 25 mL	Presence in 25 mL	560
D+2	Presence in 25 mL	Presence in 25 mL	1200
D+5	Presence in 25 mL	Presence in 25 mL	890

The results show that the *Salmonella* strain used is stable for 5 days at (4±2)°C in the pasteurized milk matrix.

4.2.3. Shipping conditions (temperature and state of the samples)

The temperatures of the samples at reception for all the collaborators are given in table 13.

Table 13: temperature and shipping conditions

Laboratory	Temperature (°C)	State of the samples
A	1,0	Correct
B	4,7	Correct
C	4,4	Correct
D	3,6	Correct
E	6,5	Correct
F	3,5	Correct
G	3,8	Correct
H	2,9	Correct
I	4,9	Correct
J	4,0	Correct
K	2,9	Correct
L	3,8	Correct
M	4,6	Correct
N	3,0	Correct

The analysis of the data from the temperature probes showed a variation between 0,5 °C and 5,2°C for all laboratories.

As a result of transport conditions, 13 laboratories carried out the tests.

4.3. Test results

The post-confirmation positive results obtained by the collaborators and by the expert laboratory are set out in the following tables. The results of the enumeration of the microorganisms of the matrix were all <10 CFU/ml.

4.3.1. Expert laboratory results

The results of the expert laboratory are summarized in table 14.

Table 14: positive results obtained by expert laboratory by both methods

Contamination level	Alternative method	Reference method
L_0	0/8	0/8
L_1	8/8	8/8
L_2	8/8	8/8

4.3.2. Collaborators results

Results of collaborators are shown in table 15 and in Appendix G.

Table 15: Positive results obtained with the reference and the alternative methods

Collaborators	Reference method			Alternative method		
	L_0	L_1	L_2	L_0	L_1	L_2
Collaborator A	0 / 8	8 / 8	8 / 8	0 / 8	8 / 8	8 / 8
Collaborator B	0 / 8	8 / 8	8 / 8	0 / 8	8 / 8	8 / 8
Collaborator C	0 / 8	8 / 8	8 / 8	0 / 8	8 / 8	8 / 8
Collaborator D	2 / 8	8 / 8	8 / 8	0 / 8	8 / 8	8 / 8
Collaborator E	0 / 8	7 / 8	8 / 8	0 / 8	7 / 8	8 / 8
Collaborator F	0 / 8	8 / 8	8 / 8	0 / 8	8 / 8	8 / 8
Collaborator G	0 / 8	8 / 8	8 / 8	0 / 8	8 / 8	8 / 8
Collaborator H	0 / 8	7 / 8	8 / 8	0 / 8	7 / 8	8 / 8
Collaborator I	0 / 8	8 / 8	8 / 8	0 / 8	8 / 8	8 / 8
Collaborator J	0 / 8	7 / 8	8 / 8	0 / 8	7 / 8	8 / 8
Collaborator K	0 / 8	8 / 8	8 / 8	0 / 8	8 / 8	8 / 8
Collaborator L	0 / 8	7 / 8	8 / 8	0 / 8	7 / 8	8 / 8
Collaborator M	0 / 8	7 / 8	8 / 8	0 / 8	7 / 8	8 / 8
Collaborator N	0 / 8	8 / 8	8 / 8	0 / 8	8 / 8	8 / 8
Total	2 / 112	107 / 112	112 / 112	0 / 112	107 / 112	112 / 112

Results are consistent with those expected for all laboratories, except for the laboratory D which found 2 samples of the reference method positive at level L_0 (samples 21 and 23). The collaborator mentioned a mistake during the sampling and performed the analyses again from the cold-stored enriched broths with both methods. The results were all negative.

According to this finding, the Expert laboratory proposed to exclude the results of laboratory D of the statistical analysis of the results. This proposition was accepted by the Technical Committee.
Final analysis was consequently conducted using data supplied by thirteen laboratories.

4.3.3. Results of the collaborators used for the statistical analysis

The results of the 13 collaborators retained for the statistical interpretation are shown in table 16.

Table 16: Positive results retained for the statistical analysis

Collaborators	Reference method			Alternative method		
	L_0	L_1	L_2	L_0	L_1	L_2
Collaborator A	0 / 8	8 / 8	8 / 8	0 / 8	8 / 8	8 / 8
Collaborator B	0 / 8	8 / 8	8 / 8	0 / 8	8 / 8	8 / 8
Collaborator C	0 / 8	8 / 8	8 / 8	0 / 8	8 / 8	8 / 8
Collaborator E	0 / 8	7 / 8	8 / 8	0 / 8	7 / 8	8 / 8
Collaborator F	0 / 8	8 / 8	8 / 8	0 / 8	8 / 8	8 / 8
Collaborator G	0 / 8	8 / 8	8 / 8	0 / 8	8 / 8	8 / 8
Collaborator H	0 / 8	7 / 8	8 / 8	0 / 8	7 / 8	8 / 8
Collaborator I	0 / 8	8 / 8	8 / 8	0 / 8	8 / 8	8 / 8
Collaborator J	0 / 8	7 / 8	8 / 8	0 / 8	7 / 8	8 / 8
Collaborator K	0 / 8	8 / 8	8 / 8	0 / 8	8 / 8	8 / 8
Collaborator L	0 / 8	7 / 8	8 / 8	0 / 8	7 / 8	8 / 8
Collaborator M	0 / 8	7 / 8	8 / 8	0 / 8	7 / 8	8 / 8
Collaborator N	0 / 8	8 / 8	8 / 8	0 / 8	8 / 8	8 / 8
Total	0 / 104	99 / 104	104 / 104	0 / 104	99 / 104	104 / 104

4.4. Calculations and interpretation

4.4.1. Calculation of the specificity

The percentage specificity (SP) of the reference method and the alternative method is calculated, using the data after confirmation, based on the results of level L_0 as follows:

- Specificity of the reference method: $SP_{ref} = \left[1 - \left(\frac{P_0}{N-}\right)\right] \times 100\%$
- Specificity of the alternative method: $SP_{alt} = \left[1 - \left(\frac{CP_0}{N-}\right)\right] \times 100\%$

where:

N- is the number of all L_0 tests,

P_0 is the total number of false-positive results obtained with the blank samples before confirmation,

CP_0 is the total number of false-positive results obtained with blank samples.

The results are the following:

- SP_{ref} = 100%
- SP_{alt} = 100%

4.4.2. Summary of the results

Table 17 details per method and per level the results obtained during the study.

*Table 17 : tests results for the two methods (PA: positive agreement, NA: negative agreement, ND: negative deviation, PD: positive deviation, PP: presumed positive before confirmation, *: for the collaborator F only with the DLIS response)*

Level	Alternative method	Reference method		
		Reference method positive (R+)	Reference method negative (R-)	Total
L0	**Alternative method positive (A+)**	PA = 0	PD = 0	0
	Alternative method negative (A-)	ND = 0 including 0 PPND	NA = 104 including 0 PPNA	104
	Total	0	104	104
L_1	**Alternative method positive (A+)**	PA = 99	PD = 0	99
	Alternative method negative (A-)	ND = 0 including 0 PPND	NA = 5 including 0 PPNA	5
	Total	99	5	104
L2	**Alternative method positive (A+)**	PA = 104	PD = 0	104
	Alternative method negative (A-)	ND = 0 including 0 PPND	NA = 0 including 0 PPNA	0
	Total	104	0	104

4.4.3. Calculation of the sensitivity of the methods, relative trueness and false positive ratio

The sensitivity of the two methods, the relative trueness and the false positive ratio parameters are calculated with the data of the table 17, according to the formulas below:

- Sensitivity for the alternative method: $SE_{alt} = \frac{(PA+PD)}{(PA+ND+PD)} \times 100\%$
- Sensitivity for the reference method: $SE_{ref} = \frac{(PA+ND)}{(PA+ND+PD)} \times 100\%$
- Relative trueness: $RT = \frac{(PA+NA)}{N} \times 100\%$
- False positive ratio for the alternative method: $FP = \frac{FP}{NA} \times 100\%$

where N is the total number of samples (NA + PA + PD + ND) and FP is false positive results.

The results are the following:

- SE_{alt} = 100%
- SE_{ref} = 100%
- RT = 100%

No false positive result was observed during this study.

4.4.4. Determination of the acceptability limit and conclusion

For a paired study, the difference between (ND – PD) and the sum of (ND + PD) is calculated. The observed values shall not be higher than the acceptability limits (AL) defined by the ISO 16140 2:2016.

The AL is not met when the observed value is higher than the AL. When the AL is not met, investigations should be made (e.g. root cause analysis) in order to provide an explanation of the observed results.

Based on the AL and the additional information, it is decided whether the alternative method is regarded as not fit for purpose. The reasons for acceptance of the alternative method in case the AL is not met shall be stated in the study report.

The different values observed are detailed in the table 18.

Table 18: values obtained for the determination of the acceptability limit

Number of collaborators	(ND-PD)	(ND+PD)	Acceptability limits (AL)	
			(ND-PD)	(ND+PD)
13	0	0	4	5

The values (ND-PD) and (ND+PD) are inferior to the AL, so the requirements of the standard ISO 16140-2: 2016 are fulfilled. The performance of the alternative method and the reference method can be considered as equivalent.

4.4.1. Evaluation of the $LOD_{50\%}$, $LOD_{95\%}$ and RLOD

The RLOD, $LOD_{50\%}$ and $LOD_{95\%}$ are calculated using the Excel spreadsheet called RLOD_inter-lab_study_16140-2_AnnexF_ver1_28_28-06-2017 available at http://standards.iso.org/iso/16140.

The values for each method are presented in table 19.

Table 19: values of LOD50% and LOD95% for reference and alternative method and value of RLOD for the alternative method (CFU/25 g)

Method	$LOD_{50\%}$	$LOD_{95\%}$	RLOD
Reference	0,69 [0,51 ; 0,91]	2,96 [2,22 ; 3,95]	1,0 [0,72 ; 1,4]
Alternative	0,69 [0,51 ; 0,91]	2,96 [2,22 ; 3,95]	

4.5. Conclusion

The data and their interpretation meet the requirements of the standard EN ISO 16140-2:2016. The performance of the alternative method and the reference method can be considered as equivalent.

5. General conclusion

The data and the interpretation of the methods comparison study and of the interlaboratory study fulfill the requirements of the standard EN ISO 16140-2:2016. The SMS method is considered as equivalent to the standard EN ISO 6579-1:2007.

Le Lion d'Angers, July 9, 2020.
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APPENDICES