The new ISO 16140 standards.

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Background (1)

- CEN-ISO standards are developed by ISO TC34/SC9 and CEN TC275/WG6.
- In 2003 the ‘old’ ISO 16140 (validation of alternative methods) was published.
- This standard was published after 10 years of development.
- Starting point was the EU (EURECA) project called Microval.
- Developed to accommodate the need for fast and reliable alternative methods.
- Popular standard, currently more than 100 alternative methods validated.
- In 2005 it became clear that there was a need to revise the 16140 and there was a need for more standards on validation.
- SC9 and WG6 decided in 2006 to set up a working group for this.
- This working group (WG3) started in 2006, under ISO SC9 lead, with the following mandate:
Background (2)

WG 3 (method validation) mandate:

2. Development standard on verification
3. Development standard on validation standardised reference methods
4. Development standard on single lab validation
5. Development standard on intermediate validation
6. Development standard on validation confirmation methods
Current status “WG3” standards (May 2019)

ISO 16140-1: Vocabulary (published July 2016)
ISO 16140-2: Protocol for the validation of alternative (proprietary) methods against a reference method (published July 2016)
ISO 16140-3: Protocol for the verification of reference and validated alternative methods implemented in a single laboratory (in preparation for FDIS vote)
ISO 16140-4: Protocol for single-laboratory (in-house) method validation (ready for FDIS vote)
ISO 16140-5: Protocol for factorial interlaboratory validation for nonproprietary methods (ready for FDIS vote)
ISO 16140-6: Protocol for the validation of alternative (proprietary) methods for microbiological confirmation and typing procedures (ready for FDIS vote)
ISO 17468: Technical requirements and guidance on establishment and revision of a standardized reference method (published July 2016)
To facilitate the selection of the appropriate standard a flow scheme is included in the introduction of the parts 3 to 6.

**Starting point is that methods should be validated.**
START: Is the method validated (performance characteristics are given)?

- **YES**
  - Is the method validated according to ISO 16140-4?
    - **YES**
      - Choose ISO 16140-2
    - **NO**
      - Apply method only in that particular laboratory (incl. scope extension)
  - Is the (food) category to be tested in the scope of the method?
    - **NO**
      - For extension of the scope of a reference method
      - For extension of the scope of an alternative (proprietary) method validated according to ISO 16140-2
      - For extension of the scope of a non-proprietary method validated according to ISO 16140-5
      - For use of the (food) type in a single laboratory, in the case of:
        - a) an alternative (proprietary) method validated according to ISO 16140-2
        - b) a non-proprietary method validated according to ISO 16140-5
        - c) a reference method with performance characteristics
        - d) a reference method without performance characteristics
      - Choose ISO 17468
    - **YES**
      - To validate alternative (proprietary) methods
      - To validate non-proprietary methods
      - To do a single-laboratory validation
      - To validate reference methods
      - Choose ISO 16140-2
      - Choose ISO 16140-5
      - Choose ISO 16140-4

- **NO**
  - Are specific (e.g. legal) requirements given to use ISO 16140 (2003) or ISO 16140-2?
    - **NO**
      - Choose ISO 16140-2
    - **YES**
      - Is the method validated according to ISO 16140-4?
        - **NO**
          - Choose ISO 16140-5
        - **YES**
          - Choose ISO 16140-4
ISO 16140-2 (validation of alternative proprietary methods)

- Basis is the comparison between a reference method and an alternative method.
- Protocol for qualitative and quantitative methods
- Both protocols have 2 phases; a method comparison study and an interlaboratory study.
- Method comparison study focusses on testing a diversity of sample/matrices (food items)
- Interlaboratory study establishes the ‘reproducibility’ of the method using a single food item.
- Evaluation of the data using preset criteria, alternative method can be better when proven.
ISO 16140-2 (validation of alternative proprietary methods)

**Qualitative study (MCS):**
- **Sensitivity study:**
  - use of (naturally) contaminated samples,
  - minimum of 5 food categories each having a minimum of 60 samples.
  - interpretation of data depending on a paired or unpaired study design.
- **RLOD study:**
  - determination of the relative level of detection using artificially contaminated samples,
  - 1 matrix per category, 20 samples per matrix.
- **Inclusivity/exclusivity study:**
  - use of 50/30 strains. *(Salmonella 100 strains)*

**Quantitative study (MCS):**
- **Relative trueness study:**
  - comparison between (naturally) contaminated samples,
  - minimum of 5 food categories each having 15 samples per category.
  - graphical interpretation of the data (Bland-Altman en scatter plots)
- **Accuracy profile study:**
  - combination of evaluation of precision and trueness (= accuracy) of the method.
  - 6 samples each with 5 replicates for each category tested.
- **Inclusivity/exclusivity study:**
  - use of 50/30 strains.
**Interlab. study** is essential in the validation of the method.

Other new aspects:
- Paired versus unpaired studies
- Acceptability Limits (AL).
- Division of matrices into categories, types and items
- Broad range of foods validation = \( \geq 5 \) food different categories

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**ISO 16140-2** (validation of alternative proprietary methods)

<table>
<thead>
<tr>
<th>Categories</th>
<th>Types</th>
<th>Items (some examples)</th>
<th>Total viable count</th>
<th>Lactic Acid bacteria</th>
<th>Yeasts/molds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pasteurized dairy products</td>
<td>Milk-based desserts, ice creams, drinks, creams</td>
<td>Y</td>
<td>Y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sterilized or UHT dairy products</td>
<td>UHT milks, canned milks or creams</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heat processed milk and dairy products</td>
<td>Fermented/acidified pasteurized milk, yoghurts, dairy-based products</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pasteurized milk products</td>
<td>Pasteurized milks, butters, creams</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hard and semi-hard cheeses (heat processed) (e.g. Comté, Emmental, Gouda)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blue cheeses (Bleu de Bresse)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Soft cheeses (e.g. Brie, Munster)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry</td>
<td>Milk powders</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Powder for milk-based desserts</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
ISO 16140-2 (validation of alternative proprietary methods)

- Part 2 focusses on the independent validation of proprietary methods.
- Validates the entire method from the food sample to (confirmed) end result.
- Procedure is important for the use of alternative methods according to European legislation (Directive 2073/2005).
- Similar to this is part 6 with the exception that this is for confirmation methods.
- So starting with a suspected colony until confirmed end result (e.g. Maldi-Toff systems).
- Focus on inclusivity/exclusivity with number of strains dependent on level of confirmation (genus or subspecies, ..).
ISO 16140-3 (method verification) (1)

Definitions

› **Validation**: establishment of the performance characteristics of a method and provision of objective evidence that the performance requirements for a specified intended use are fulfilled.

› **Verification**: demonstration that a validated method functions in the user’s hands according to the method’s specifications determined in the *validation* study and is fit for its purpose.

Part 3 is only applicable to validated alternative or reference methods!
### Parameters to be determined in verification.

<table>
<thead>
<tr>
<th>Method</th>
<th>Performance characteristic</th>
<th>Implementation Verification</th>
<th>(Food) item verification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qualitative</td>
<td>Estimated LOD$<em>{50}$ (eLOD$</em>{50}$)</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Quantitative</td>
<td>Intralaboratory reproducibility standard deviation (S$_{IR}$)</td>
<td>✓</td>
<td>Not applicable</td>
</tr>
<tr>
<td></td>
<td>Estimated bias (eBias)</td>
<td>Not applicable</td>
<td>✓</td>
</tr>
</tbody>
</table>
**ISO 16140-3 (method verification) (3)**

**Estimated LOD<sub>50</sub>**

- Three protocols available.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Inoculation level of the test portion</th>
<th>Blank</th>
<th>Total number of replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9 × LOD&lt;sub&gt;50&lt;/sub&gt;/test portion</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 × LOD&lt;sub&gt;50&lt;/sub&gt;/test portion</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 × LOD&lt;sub&gt;50&lt;/sub&gt;/test portion</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 to 5 cfu/test portion</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blank</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total number of replicates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Estimated LOD$_{50}$

- Criterium $eLOD \leq 4 \times LOD_{50}$
- Example for 3 dilutions

Most alternative methods do not have LOD$_{50}$ data but RLOD!

ISO 16140-3 (method verification) (4)

<table>
<thead>
<tr>
<th>High inoculation level</th>
<th>Intermediate inoculation level</th>
<th>Low inoculation level</th>
<th>Blank level</th>
<th>eLOD$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>= 18 cfu/test portion</td>
<td>= 6 cfu/test portion</td>
<td>= 2 cfu/test portion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/1</td>
<td>4/4</td>
<td>4/4</td>
<td>0/1</td>
<td>&lt; 2,0</td>
</tr>
<tr>
<td>1/1</td>
<td>4/4</td>
<td>3/4</td>
<td>0/1</td>
<td>= 1,0</td>
</tr>
<tr>
<td>1/1</td>
<td>4/4</td>
<td>2/4</td>
<td>0/1</td>
<td>= 1,4</td>
</tr>
<tr>
<td>1/1</td>
<td>4/4</td>
<td>1/4</td>
<td>0/1</td>
<td>= 2,0</td>
</tr>
<tr>
<td>1/1</td>
<td>4/4</td>
<td>0/4</td>
<td>0/1</td>
<td>= 3,0</td>
</tr>
</tbody>
</table>
**ISO 16140-3 (method verification) (5)**

**Estimated bias (eBias)**

- Criterium: $eBias \leq 0.5 \log$

<table>
<thead>
<tr>
<th>Sample</th>
<th>Test portion</th>
<th>Artificially contaminated (food) item</th>
<th>Inoculum suspension [without (food) item]</th>
<th>Absolute difference in results between artificially contaminated (food) item and the inoculum suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1, test portion 1</td>
<td></td>
<td>2.06 (average of 1.87 and 2.25)</td>
<td>2.17</td>
<td>−0.11</td>
</tr>
<tr>
<td>Sample 1, test portion 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample 2, test portion 1</td>
<td></td>
<td>3.99 (average of 3.93 and 4.04)</td>
<td>4.29</td>
<td>−0.30</td>
</tr>
<tr>
<td>Sample 2, test portion 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample 3, test portion 1</td>
<td></td>
<td>3.68</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
ISO 16140-3 (method verification) (6)

**Intralab. Reproducibility ($S_{IR}$)**

- Link with ISO 19036 (2019)
- Is the technical uncertainty as in ISO 19036
ISO 16140-3 (method verification) (7)

**Intralab. Reproducibility ($S_{IR}$)**

- Criterium: $S_{IR} \leq 2 \times S_R$
  (lowest observed mean)

<table>
<thead>
<tr>
<th>(Food) item</th>
<th>$S_R$ values from the validation study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low level</td>
</tr>
<tr>
<td>Liquid pasteurized egg</td>
<td>0,32</td>
</tr>
<tr>
<td>Minced meat</td>
<td>0,28</td>
</tr>
<tr>
<td>(Animal) feed</td>
<td>0,18</td>
</tr>
<tr>
<td>Pasteurized milk</td>
<td>0,24</td>
</tr>
<tr>
<td>Tiramisu</td>
<td>0,22</td>
</tr>
</tbody>
</table>
ISO 16140-3 (method verification) (8)

- Also part on verification of validated confirmation methods
  - 5 strains inclusivity
  - 5 strains exclusivity

- General: When results do not comply → **Root cause analysis!!!**

- What to do with non-validated reference methods?
- What to do with existing methods?
A guidance document is drafted in order to guide:
- user laboratories,
- (technical) assessors,
- accreditation bodies but also
- regulatory agencies
how to implement 16140-3.

Main points:
- Crucial for the acceptance of part 3 is the lack of requirement to repeat verification/validation of existing methods!
- In addition a transition period (until 1-1-2027) is included to be able to verify non-validated reference (ISO/CEN) standard methods.
A guidance document is drafted in order to guide user laboratories, technical assessors, accreditation bodies, but also regulatory agencies, how to look upon the implementation of 16140-3.

Crucial for the acceptance of part 3 is the lack of requirement to redo verification/validation of existing methods!

In addition a transition period (until 1-1-2027) is included to be able to verify non-validated reference (ISO/CEN) standard methods.
Other parts

**Part 4: single lab validation**
- Validation of a method against or without a reference method
- Based on traditional way of validation and factorial design validation
- Part on validation of confirmation methods
- **Results only valid for the lab that did the validation!**

**Part 5: Factorial design ILS**
- Alternative design for interlaboratory study using less laboratories.
- No to replace ILS from 16140-2.

**Part 7: …….
Thank you for your attention

Questions?????