Validation of alternative microbiological methods - the ISO 16140 series.

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ISO TC 34/SC 9/WG 3

• 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.
• 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 decided in 2006 to set up a working group for this.
• This working groups started in 2006 with the following mandate:
ISO TC 34/SC 9/WG 3

WG 3 (method validation) mandate:
- Development standard on verification
- Development standard on validation standardised reference methods
- Development standard on single lab validation
- Development standard on intermediate validation
- Development standard on validation confirmation methods
Standards (to be) published prepared by WG3

ISO 16140-1: Vocabulary (published)
ISO 16140-2: Protocol for the validation of alternative (proprietary) methods against a reference method (published)
ISO 16140-3: Protocol for the verification of reference and validated alternative methods implemented in a single laboratory (in preparation for DIS vote)
ISO 16140-5: Protocol for factorial interlaboratory validation for nonproprietary methods (in preparation for DIS vote)
ISO 16140-6: Protocol for the validation of alternative (proprietary) methods for microbiological confirmation and typing procedures (in preparation for DIS vote)
ISO 17468: Technical requirements and guidance on establishment or revision of a standardized reference method (published)
START: Is the method validated (performance characteristics are given)?

- NO
  - Is the method a standardized reference method?
    - NO
      - Are specific (e.g. legal) requirements given to use ISO 16140 (2003) or ISO 16140-2?
        - NO
          - To validate alternative (proprietary) methods
            - Choose ISO 16140-2
          - To validate alternative methods
            - Choose ISO 16140-5
        - YES
          - Choose ISO 16140-2
    - YES
      - Is the alternative method validated according to ISO 16140-4?
        - NO
          - Regard the method as not validated and go back to start
        - YES
          - Apply method only in that particular laboratory (incl. scope extension)

- YES
  - Is the method a standardized reference method or a method validated according to ISO 16140-2 or ISO 16140-5?
    - NO
      - Is the (food) type to be tested in the scope of the method?
        - NO
          - Choose ISO 16140-3 (verification)
        - YES
          - Is the (food) type to be tested in the scope of the method?
            - NO
              - a] for an extension of the scope of a standardized reference method or
                - b] for a complete validation of a reference method
                - Choose ISO 17468
            - YES
              - For an extension of the scope of an alternative (proprietary) method, validated according to ISO 16140-2
                - Choose ISO 16140-2
              - For an extension of the scope of an alternative method, validated according to ISO 16140-5
                - Choose ISO 16140-5
              - For use of the (food) type in a single laboratory, in case of:
                - a] an alternative (proprietary) method validated according to ISO 16140-2 or
                - b] an alternative method validated according to ISO 16140-5 or
                - c] a standardized reference method with performance characteristics or
                - d] a standardized reference method without performance characteristics
                  - 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 samples/matrices
- Interlaboratory study establishes the ‘reproducibility’ of the method using a single matrix.
- 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, comparison between (naturally) contaminated samples, minimum of 5 food categories each having a minimum of 60 samples. Separation in interpretation of data from a paired and unpaired study design.
- RLOD study, determination of the relative level of detection using artificially contaminated samples, 1 matrix per category, 30 samples per matrix.
- Inclusivity/exclusivity study using 50/30 strains.
ISO 16140-2 (Validation of alternative (proprietary) methods)

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. Six samples each with 5 replicates for each category tested.
- Inclusivity/exclusivity study using 50/30 strains.
ISO 16140-3 (Verification of methods)

- 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.

- Still heavily debated!
### 16140-3 (Verification of methods)

<table>
<thead>
<tr>
<th>Method with published validation data</th>
<th>Method without published validation data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference method</td>
<td>Alternative method</td>
</tr>
<tr>
<td>Implementation verification</td>
<td>✓</td>
</tr>
<tr>
<td>(Food) type verification</td>
<td>✓</td>
</tr>
</tbody>
</table>

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*a* Not applicable: the method first needs to be validated (see Figure 1).
ISO 16140-3 (Verification of methods)

Current draft:
- Qualitative methods: estimated LOD$_{50}$
- Quantitative methods: estimated bias en reproducibility ($S_{IR}$)
  (link with measurement uncertainty ISO 19036)
Table 3 — Estimation of $LOD_{50}$ based on the number of positive results per level of contamination

<table>
<thead>
<tr>
<th>High level of inoculation (ca. $10 \times LOD_{50}$ /test portion)</th>
<th>Intermediate level of inoculation (ca. $5 \times LOD_{50}$ /test portion)</th>
<th>Low level of inoculation (ca. $1 \times LOD_{50}$ /test portion)</th>
<th>Blank control</th>
<th>Estimated $LOD_{50}$ (cfu/test portion)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2/2</td>
<td>2/2</td>
<td>2/2</td>
<td>0/2</td>
<td>&lt; $1 \times LOD_{50}$</td>
</tr>
<tr>
<td>2/2</td>
<td>2/2</td>
<td>1/2</td>
<td>0/2</td>
<td>= $1 \times LOD_{50}$</td>
</tr>
<tr>
<td>2/2</td>
<td>2/2</td>
<td>0/2</td>
<td>0/2</td>
<td>= $2 \times LOD_{50}$</td>
</tr>
<tr>
<td>2/2</td>
<td>1/2</td>
<td>2/2</td>
<td>0/2</td>
<td>= $2 \times LOD_{50}$</td>
</tr>
<tr>
<td>2/2</td>
<td>1/2</td>
<td>1/2</td>
<td>0/2</td>
<td>= $3 \times LOD_{50}$</td>
</tr>
<tr>
<td>2/2</td>
<td>1/2</td>
<td>0/2</td>
<td>0/2</td>
<td>= $4 \times LOD_{50}$</td>
</tr>
<tr>
<td>2/2</td>
<td>0/2</td>
<td>2/2</td>
<td>0/2</td>
<td>Unreliable result a</td>
</tr>
<tr>
<td>2/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>= $5 \times LOD_{50}$</td>
</tr>
<tr>
<td>2/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>= $8 \times LOD_{50}$</td>
</tr>
</tbody>
</table>

*a This combination of results is very unlikely to occur with the levels of contamination tested and therefore leads to an unreliable result. The test shall therefore be repeated.
## Table 5 — Test results

<table>
<thead>
<tr>
<th>Method to be verified</th>
<th>Reference method</th>
<th>Method to be verified</th>
<th>Reference method</th>
<th>Difference between alternative and reference method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch 1, test portion 1</td>
<td>1,87</td>
<td>2,15</td>
<td>2,06</td>
<td>2,17</td>
</tr>
<tr>
<td>Batch 1, test portion 2</td>
<td>2,25</td>
<td>2,20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Batch 2, test portion 1</td>
<td>3,93</td>
<td>4,23</td>
<td>3,99</td>
<td>4,29</td>
</tr>
<tr>
<td>Batch 2, test portion 2</td>
<td>4,04</td>
<td>4,35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Batch 3, test portion 1</td>
<td>3,73</td>
<td>3,59</td>
<td>3,68</td>
<td>3,62</td>
</tr>
<tr>
<td>Batch 3, test portion 2</td>
<td>3,63</td>
<td>3,65</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
ISO 16140-4 (Single-lab validation)

• Experimental design two-fold: classical approach and factorial design approach.
• For both experimental designs a protocol is described with and without the use of a reference method (using a reference value).
• Separate protocols for qualitative and quantitative methods
• Results of the validation study are only valid in the laboratory that conducted the study!
ISO 16140-5 (factorial interlab validation)

- Factorial interlaboratory study.
- By using the ‘factorial design’ less laboratories (≤ 4) are needed for the study (in comparison to 16140-2).
- Factorial approach cannot replace the interlaboratory study of an alternative (proprietary) method according to 16140-2.
ISO 16140-6 (validation confirmation methods)

- Validation of a part or complete confirmation procedure of a reference method against a (proprietary) alternative confirmation method (e.g. API 20 E or PCR test or Maldi-TOF).
- Validation starts with a suspected colony and not a (food) sample.
- Is based on the inclusivity/exclusivity study of 16140-2, using well characterised strains.
- Differentiation between validation at genus, species or subspecies level.
- Comparison between reference method and alternative method is basis. Comparison between identity of strain only done in cases where differences occur.
- Has both method comparison study and interlab study (use of reference method is optional for interlab study)
START: Is the method validated (performance characteristics are given)?

- **YES**
  - Is the method a standardized reference method?
    - **YES**
      - Are specific (e.g. legal) requirements given to use ISO 16140 (2003) or ISO 16140-2?
        - **NO**
          - To validate alternative methods
            - Choose ISO 16140-2
        - **YES**
          - To do a single-laboratory validation
            - Choose ISO 16140-5
    - **NO**
      - To validate alternative methods
        - Choose ISO 16140-5
  - **NO**
    - Regard the method as not validated and go back to start

- **NO**
  - Is the method a standardized reference method or a method validated according to ISO 16140-2 or ISO 16140-5?
    - **YES**
      - Is the method validated according to ISO 16140-4?
        - **NO**
          - Regard the method as not validated and go back to start
        - **YES**
          - Apply method only in that particular laboratory (incl. scope extension)
            - Choose ISO 16140-2
  - **NO**
    - Is the (food) type to be tested in the scope of the method?
      - **YES**
        - Choose ISO 16140-3 (verification)
      - **NO**
        - Is the alternative method validated according to ISO 16140-2 or ISO 16140-5?
          - **YES**
            - Choose ISO 16140-2
          - **NO**
            - Regard the method as not validated and go back to start

For an extension of the scope of a standardized reference method or a complete validation of a reference method:
- Choose ISO 17468
- Choose ISO 16140-2
- Choose ISO 16140-5
- Choose ISO 16140-4

For an extension of the scope of an alternative (proprietary) method, validated according to ISO 16140-2:
- Choose ISO 16140-2
- Choose ISO 16140-5
- Choose ISO 16140-4

For an extension of the scope of an alternative method, validated according to ISO 16140-5:
- Choose ISO 16140-5
- Choose ISO 16140-4

For use of the (food) type in a single laboratory, in case of:
- a) an alternative (proprietary) method validated according to ISO 16140-2 or ISO 16140-5 or a standardized reference method with performance characteristics or a standardized reference method without performance characteristics:
  - Choose ISO 16140-4

DRAFT Figure - Flow chart for the ISO 16140-series Date: 21 March 2016