The Certification of a new set of Reference Materials of Soya Powder with different Mass Fractions of Roundup Ready™ Soya

Certified Reference Materials IRMM-410R*

(SB-0 / SB-0.1 / SB-0.5 / SB-1 / SB-2 / SB-5)

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SUMMARY

This report describes the preparation and certification of a new set of six soya bean powder CRM's (IRMM-410R) with different mass fractions of genetically modified Roundup Ready™ dry powder (Certified reference materials SB-0, SB-0.1, SB-0.5, SB-1, SB-2 and SB-5). The CRM's were produced by IRMM on behalf of Fluka Chemie AG in replacement of IRMM-410, produced in 1999 in the frame of a project of the Environment Institute (EI) of the Joint Research Centre of the European Commission (Ispra, Italy) aiming at the validation of the polymerase chain reaction (PCR) screening method for the detection of genetically modified plants. The Roundup Ready™ concentration of IRMM-410R was verified at Kantonales Laboratorium Basel-Stadt in Switzerland. The CRM's are available in the form of glass bottles containing 1 g of soya beans powder packed under argon atmosphere. Seeds of biologically grown non-modified soya bean and Roundup Ready™ GMO soya bean were heated at 90°C for 17 to 35 h in order to reduce enzymatic activities and ground and dried afterwards. The materials were prepared by quantitative mixing in aqueous suspension of non-modified soya bean powder and 100 % Roundup Ready™ GMO soya bean powder, and subsequent freeze-drying, grinding, homogenisation and bottling. The pre-filled bottles were submitted to a supplemental primary vacuum drying for around 2 h and closed after filling with pure argon. It is recommended to use samplings not smaller than 100 mg. Unopened bottles can be stored at room temperature, preferably in the dark. Once opened, bottles should be stored dry and cool (4 °C).
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1. Introduction

Genetical modification of agricultural products is becoming an increasingly important item and authorisations to market such products are more and more under discussion\(^1\). However, in the EU such authorisations are being combined with the obligation to declare food containing ingredients from genetically modified plants. This enforces the necessity to develop suitable detection methods, and subsequently to dispose of certified reference materials. Therefore, the preparation of CRM's of mixtures of genetically modified (GMO) and non-genetically modified organisms has been started to allow proper validation of screening methods for the detection of genetic modification in agricultural and food products. A set of CRM's of soya bean powder with different mass fractions (0, 0.1, 0.5, 1, 2, 5 \%) of dried powder of genetically modified (Roundup Ready\textsuperscript{TM}) soya beans was produced by IRMM on behalf of Fluka Chemie AG, Buchs, Switzerland. This CRM IRMM-410R is replacing IRMM-410, which was produced in two series leading to different intensities of DNA degradation. IRMM-410R, produced in one series, proofed to be a consistent set. The six CRM's (SB-0, SB-0.1, SB-0.5, SB-1, SB-2 and SB-5) are available both from Fluka Chemie AG (vials with Fluka label) and from IRMM (vials with IRMM label). Both products are however identical and distributed with an IRMM Certificate.

2. CRM Preparation

2.1 Starting materials

For the preparation of the CRM's, 45 kg Roundup Ready\textsuperscript{TM} soya beans (Roundup Ready is a trademark of Monsanto Company) and 200 kg non-GMO soya beans grown in a biological farm were supplied to IRMM by Fluka Chemie AG, Buchs. The delivered materials were dried in air on stainless steel trays at 90 \(\text{°C}\) for 17 to 35 hours. After this heat treatment, the initial moisture content of the base materials (7 - 10 \%) was decreased to less than 2 \%. The dried starting materials were then ground using a Fine-Impact Mill 100 UPZ (Alpine, Augsburg, DE) equipped with a fan beater unit and an open grinding track. After completion of the fine grinding, the ground materials (195 kg non-GMO, 42 kg GMO) were collected in polyethylene containers.

2.2 The quantitative preparation of GMO/non-GMO mixtures

Mixtures containing 0.1, 0.5, 1, 2, and 5 \% GMO soya powder and the 0 \% GMO soya powder were prepared quantitatively using a suspension method. Water contents of the dried GMO and non-GMO powders, used as base materials, were determined threefold by Karl Fischer titration (758 KFD Titrino, Methrom, Herisau, CH) in order to correct for the water content of the material. Preparation details are given in Table 1. Afterwards powders were weighed using calibrated balances. A 10 l container was used to prepare the different batches and was placed in an outer container filled with cooling water, using a cooling device (Haake C40 / F6 from Karlsruhe, DE) to lower production temperature. First the inner container was filled with appropriate quantities of cold (+4 \(\text{°C}\)), demineralised water; afterwards the manually premixed GMO and non-GMO fraction was added subsequently. The mixture was stirred for two hours with a Turbotron mixer equipped with a ringed paddle stirring device (Janke & Kunkel, Staufen, DE) at approximately 400 rpm. During the mixing time the temperature decreased from +7 to +3 \(\text{°C}\). Afterwards the suspension was put into trays for freeze-drying.
The standard uncertainty on the dried GMO powder mass fractions of the CRM preparations (i.e. 0.1, 0.5, 1, 2 and 5 % GMO) is estimated at 0.1 % relative.

<table>
<thead>
<tr>
<th>Mixture</th>
<th>Water content of base material</th>
<th>Mass after water correction</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMO (%)</td>
<td>GMO (%)</td>
<td>Non-GMO (%)</td>
</tr>
<tr>
<td>0</td>
<td>n.a.</td>
<td>3.90</td>
</tr>
<tr>
<td>0.1</td>
<td>2.69</td>
<td>4.32</td>
</tr>
<tr>
<td>0.5</td>
<td>2.69</td>
<td>4.32</td>
</tr>
<tr>
<td>1</td>
<td>2.80</td>
<td>4.42</td>
</tr>
<tr>
<td>2</td>
<td>2.80</td>
<td>4.42</td>
</tr>
<tr>
<td>5</td>
<td>2.69</td>
<td>4.32</td>
</tr>
</tbody>
</table>

Table 1. Details of the preparation of various GMO/non-GMO mixtures for freeze-drying of soya bean powder

2.3 Freeze-drying
Freeze-drying was carried out using an Epsilon freeze-dryer D85-2 (M. Christ, Osterode, DE) with a shelf capacity of 5.4 m². The freeze-drying programme consisted of a 24 h primary drying at −10 °C, followed by a 12 h drying at 0 °C and, finally, a 48 h secondary drying at +20 °C. The 300 mm x 450 mm trays were each loaded with 1.1 kg of suspension. 220 g of a hard cake per kg of suspension were obtained after freeze-drying.

2.4 Grinding, homogenisation and bottling
The freeze-dried products were subsequently ground using a Fine-Impact Mill 100 UPZ (Alpine, Augsburg, DE) in the same way as described under 2.1. Subsequently, they were homogenised for 2 hours using a Turbula mixer and bottled in well cleaned 10 ml brown glass vials using an automatic sampling device DMR 1 (Transmatic Fyllan Limited, Bedford, UK). Before sealing the vials with aluminium caps, they were put into the freeze-dryer for a final primary vacuum drying for 1 to 3 h, depending on the actual water content of the product. Bottles were filled with argon and closed with rubber stoppers using the hydraulically moveable device of the freeze-dryer.

2.5 Production control
The moisture content of the dry powders was determined by Karl Fischer titration and amounted typically to values in the range of 2.2 to 2.7 %. Particle size measurements of the various powders were carried out using a particle size analyser with Helos measuring device (Sympatec, Clausthal-Zellerfeld, DE). After grinding, homogenisation and bottling of the freeze-dried cakes, the powders had a particle size typically < 540 μm and an average particle size between 140 and 190 μm.

2.6 Minimum sample intake for PCR analysis
As no sufficiently precise methods exist to determine the different DNA sequences quantitatively, it was not possible to perform a real homogeneity testing of the certified mixtures. However, the mixing of flours in aqueous suspension with the help of a ringed
paddle stirring device has been found to lead to sufficient homogeneity, provided that a sufficient number of particles are used for each analysis. For the recommended sample intake of 100 mg per analysis, one may estimate on the basis of the particle size distribution of the mixtures that this number is >> 10^4, which means that even for SB-0.1 the number of GMO particles is >> 50. On this basis uncertainties due to sample inhomogeneity were estimated (see table 2).

3. **Certified mass fractions of GMO**

The materials SB-0, SB-0.1, SB-0.5, SB-1, SB-2 and SB-5 form a set of 6 reference materials certified for the mass fraction of dried Roundup Ready™ soya bean powder in dried soya bean powder. The certified mass fractions are based on quantitative mixing in aqueous suspension of biologically grown non-modified dried soya bean powder with dried Roundup Ready™ soya bean powder. Referring to the particle size distribution (see 2.6) it is advised to use sample intakes not smaller than 100 mg. Taking into account the uncertainties of the weighing and of the humidity contents of the starting materials, uncertainties for the certified mass fractions at 100 mg level were estimated (see Table 2).

It must however be emphasised that due to the relatively large uncertainty inherent to quantitative DNA determinations, the DNA/dry powder mass fraction of different lots of soya beans cannot be determined with the highest precision. Therefore, the ratio GMO-DNA/non-GMO-DNA in the reference materials may significantly deviate from the certified powder mass ratio values.

<table>
<thead>
<tr>
<th>CRM</th>
<th>Certified</th>
<th>Standard Uncertainty</th>
<th>Combined uncertainty</th>
<th>Expanded uncertainty</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(u_x)</td>
<td>(u_y)</td>
<td>(u_z)</td>
</tr>
<tr>
<td>SB-0</td>
<td>0.000</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>SB-0.1</td>
<td>0.100</td>
<td>0.0001</td>
<td>0.001</td>
<td>0.013</td>
</tr>
<tr>
<td>SB-0.5</td>
<td>0.50</td>
<td>0.0005</td>
<td>0.005</td>
<td>0.030</td>
</tr>
<tr>
<td>SB-1</td>
<td>1.00</td>
<td>0.001</td>
<td>0.01</td>
<td>0.042</td>
</tr>
<tr>
<td>SB-2</td>
<td>2.00</td>
<td>0.002</td>
<td>0.02</td>
<td>0.060</td>
</tr>
<tr>
<td>SB-5</td>
<td>5.00</td>
<td>0.005</td>
<td>0.05</td>
<td>0.094</td>
</tr>
</tbody>
</table>

Table 2. Uncertainty estimation (in g GMO dry powder per 100 g dry powder)

4. **Verification of Roundup Ready™ mixtures**

4.1 **Verification study**

All six materials were analysed at Kantonales Laboratorium Basel-Stadt in Switzerland, using the following RT-PCR methods for the quantification of the GMO content (9):
Soya specific lectine real-time PCR (lectine-RT-PCR)

- Primer:
  
  | TM-Lectin-F | 5'-tcc acc ccc atc cac att t-3' |
  | TM-Lectin-R | 5'-ggc ata gaa ggt gaa gtt gaa gga-3' |

- Probe:
  
  | Lectin-FAM | 5'-(FAM)-aac cgg tag cgt tgc cag ctt cg-(TAMRA)-3' |

GMO screening method on 35S promoter (35S-prom.-RT-PCR)

- Primer:
  
  | TM-35S-1 | 5'-gcc tct gcc gac agt ggt-3' |
  | TM-35S-2 | 5'-aag acg tgg tga cgt ctt c-3' |

- Probe:
  
  | 35S-FAM | 5'-(FAM)-caa aga tgg acc ccc acc cac g-(TAMRA)-3' |

Roundup Ready™ specific real time PCR (RRS-soya-RT-PCR)

- Primer:
  
  | RRS-F | 5'-gcc atg tgt tta att tgt gcc at-3' |
  | RRS-R | 5'-gaa gtt cat ttc att tgt aga gga c-3' |

- Probe:
  
  | RRS-FAM | 5'-(FAM)-ctt gaa aga tct gct aga gtc tga ggc g-(TAMRA)-3' |

The verification study was carried out using a soya specific lectine real-time PCR (lectine-RT-PCR) to determine the soya concentration. The screening method for the identification of genetically modified organisms (GMO) in food is based on the detection of the CAMV 35S promoter by means of a real time polymerase chain reaction (RT-PCR). The specific detection of Roundup Ready™ soya can be carried out with primers binding to the Roundup Ready™ gene. All three RT-PCR methods were carried out by P. Brodmann (Kantonales Laboratorium Basel-Stadt, Basel [CH]) and are based on the extraction of DNA from the sample material and the amplification of specific DNA-sequences with appropriate primers. The amplified PCR products are measured cycle-by-cycle with target specific reporter and quencher dyes, which lead to an increased fluorescence. The number of cycles (Ct-value) which are required to pass a fluorescence threshold correlates with the amount of target DNA in the starting sample.

4.2 Results

Quantification of the GMO content of six mixtures of Roundup Ready™ soya bean powders proved the consistency of CRM IRMM-410R (table 3). All results were calibrated against 100% GMO RR-soya base material in order to allow comparison of the different concentrations. Each concentration was extracted four times and determined twice. Samples have been analysed for their quantity of 35S promoter. The screening method verified the GMO content of CRM IRMM-410R SB-0, SB-0.1, SB-0.5, SB-1, SB-2 and SB-
5 if calibrated with 100% GMO RR-soya. The quantification of specific Roundup Ready™ DNA, indicating the transition 35S-EPSPS gene, confirmed the verified GMO content. The quantification of 35S promoter and the quantification of Roundup Ready™ DNA led to comparable results proving that DNA degradation that might have occurred during production of IRMM-410R did not effect the relation of the two target sequences.

<table>
<thead>
<tr>
<th>CRM</th>
<th>Certified GMO concentration (%)</th>
<th>Lectine-RT-PCR</th>
<th>35S-Prom. RT-PCR</th>
<th>RRS-Soja RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 % RR-soya (%)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>100 ± 26</td>
<td>103.4 ± 20</td>
<td>101.2 ± 15</td>
<td></td>
</tr>
<tr>
<td>SB-0</td>
<td>0</td>
<td>162 ± 25</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>SB-0.1</td>
<td>0.10 ± 0.03</td>
<td>141 ± 21</td>
<td>0.1 ± 0.01</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>SB-0.5</td>
<td>0.50 ± 0.06</td>
<td>119 ± 41</td>
<td>0.5 ± 0.2</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>SB-1</td>
<td>1.0 ± 0.1</td>
<td>107 ± 33</td>
<td>0.9 ± 0.3</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>SB-2</td>
<td>2.0 ± 0.2</td>
<td>129 ± 18</td>
<td>2.3 ± 0.5</td>
<td>2.7 ± 0.7</td>
</tr>
<tr>
<td>SB-5</td>
<td>5.0 ± 0.2</td>
<td>98 ± 20</td>
<td>5.2 ± 1.1</td>
<td>6.4 ± 1.6</td>
</tr>
</tbody>
</table>

RT Real Time
PCR Polymerase Chain Reaction
<sup>1</sup> 100 % base material (heated, ground, not lyophilised) used for calibration

Table 3. Quantification of GMO content

5. References

(2) Molekularbiologische Methoden (in press), in: Swiss Food Manual (Schweizerisches Lebensmittelbuch), Bundesamt für Gesundheit (eds.), Eidgenössische Drucksachen- und Materialzentrale, Bern
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