



Draft Assessment Report

Evaluation of Active Substances

Plant Protection Products

Prepared according to **Regulation (EC) 1107/2009**
as it applies in Great Britain

Elemental iron

Volume 3 – B.5 (AS)

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B.5. METHODS OF ANALYSIS

B.5.1. METHODS USED FOR THE GENERATION OF PRE-AUTHORISATION DATA

B.5.1.1. Methods for the analysis of the active substance as manufactured

B.5.1.1.1. Active substance in the active substance as manufactured

Report: ██████████ (2018) Validation of the Method of Determination of the Active Ingredient and Specified Impurities in Iron Powder Technical Material, in Compliance with Good Laboratory Practice. Report number: DNA4143, Sponsor Reference number: R-39836

Previous evaluation

None.

Iron

Sample preparation: The assay of iron was performed using approximately 0.1 g of each sample of technical material in duplicate. The mass of the technical material was accurately recorded, transferred to a digestion vessel and made up in 5 mL of nitric acid and 5 mL of hydrochloric acid. The samples were then digested in a microwave digester for 55 minutes and the 10 mL digested sample was transferred to a 100 mL volumetric flask and made to volume with deionised water. These solutions were further diluted 1:100 and used for assay by injecting each solution once into the ICP-OES under the following conditions:

ICP-OES Conditions:

Instrument: Perkin Elmer ICP-OES
 Mode: Axial
 Gas Flow: Plasma: 15 mL/minute; Auxiliary: 0.2 mL/minute; Nebulizer: 0.8 mL/minute
 Pump Flow Rate: 1.0 mL/minute
 RF Power: 1300 watts
 Wavelength: Iron: 273.955 nm
 Data Acquisition: Win Lab

Microwave Digester Conditions:

Instrument: Milestone Ethos EZ

Time (minutes)	Temperature (°C)	Power (Watts)
0-15	0-100	1200
15-35	100	1200
35-55	Cooling period	0

A summary of the validation data are given in Table 1.

Table 1 Summary of method validation for method validated in study DNA4143 for the determination of iron content in elemental iron technical material

Matrix	Analyte	LOQ (g/kg)	Recovery fortification level (g/kg)	Recoveries (%) (mean, n)	Repeatability % RSD (n)	Linearity	Specificity
Elemental Iron Technical Material	Iron	100	100 1000	97.18 – 103.4 (100.4, 6) 98.04 – 101.0 (99.81, 6)	0.317 (6) at 992 g/kg Modified Horwitz: 1.342 Horrat $H_r = 0.236$	0.5 – 20 mg/L (50 – 2000 g/kg) (n=6x2) $r^2 = 0.9993$ Equation: $y = 0.00002x + 0.1508$	The ICP-OES method is highly specific to the analyte. Samples are analysed using the elements characterised emission wavelength. No interference with the solvent blank or other impurities was observed.

The determination of the LOQ or recovery is not technically required for the active substance in the technical material. However these results have been reported for completeness.

Batch no. 'DNA4043/1' was used to determine the method precision. No significant interference is observed between any of the analytes and the active substance; the analytical method used is considered highly specific to the analyte of interest. A certified reference standard and library of elemental wavelengths confirmed the identity of iron and the highly specific nature of this method. The Horrat value determined is below the acceptable limit (≤ 1). The linear range extends over an appropriate range considering the level of active substance in the proposed specification.

The method of analysis is considered fully validated in accordance with both SANCO/3030/99 rev. 4 and rev. 5.

Applicability of existing CIPAC Method

CIPAC methods for the determination of 'elemental iron' in technical material and formulation are not currently available. However, there are CIPAC methods available for the determination of total iron and divalent iron; CIPAC Method MT 95.

The technical material is a material certified as "Food Grade Quality" according to Food Chemical Codex (FCC 2016) and is sold to be used as nutritional supplement. Following the FCC, only 3 relevant impurities, Arsenic, Lead and Mercury, have been specified in the specification.

The Food Chemicals Codex (FCC) provides a collection of internationally recognized standards for the purity and identity of food ingredients. Some methods are available for iron, [REDACTED]. The FCC has been published since 1966. It provides essential criteria and analytical methods to authenticate and determine the quality of food ingredients.

Report:	Anonymous (1981) Third Edition “Food Chemicals Codex”, page 151.
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Previous evaluation	None.
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Determination of iron content:

Iron [REDACTED]: Transfer about 200 mg, accurately weighed, into a 300 mL Erlenmeyer flask, add 50 mL of diluted sulfuric acid TS, and close the flask with a stopper containing a Bunsen valve, made by inserting a glass tube connected to a short piece of rubber tubing with a slit on the side and a glass rod inserted in the other end and arranged so that gases can escape but air cannot enter. Heat on a steam bath until the iron is dissolved, cool the solution, dilute it with 50 mL of recently boiled and cooled water, add 2 drops of orthophenanthroline TS, and titrate with 0.1 N ceric sulfate until the red color changes to a weak blue. Each mL of 0.1 N ceric sulfate is equivalent to 5.585 mg of Fe.

Iron [REDACTED] The [REDACTED] iron is dissolved in dilute mineral acids with the evolution of hydrogen and the formation of solutions of the corresponding salts, which gives positive test for Ferrous Salts.

These methods have not been considered further as a fully validated method for the determination of iron content in the technical material has been reported ([REDACTED] 2018). These methods were reported by the applicant so have been reported for completeness.

B.5.1.1.2. Relevant impurities in the active substance as manufactured

Report:	[REDACTED] (2018) Validation of the Method of Determination of the Active Ingredient and Specified Impurities in Iron Powder Technical Material, in Compliance with Good Laboratory Practice. Report number: DNA4143, Sponsor Reference number: R-39836
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Previous evaluation	None.
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Arsenic

Sample preparation: The assay of arsenic was performed using approximately 2.5 g of technical material. The mass of the technical material was accurately recorded, transferred to a digestion vessel and made up in 5 mL of nitric acid and 5 mL of hydrochloric acid. The samples were then digested in a microwave digester for 55 minutes and the 10 mL digested sample was transferred to a 50 mL volumetric flask and made to volume with deionised water. These samples were used for assay by injecting each solution once into the ICP-OES under the following conditions:

ICP-OES Conditions:

Instrument: Perkin Elmer ICP-OES
 Mode: Axial
 Gas Flow: Plasma: 15 mL/minute; Auxiliary: 0.2 mL/minute; Nebulizer: 0.8 mL/minute
 Pump Flow Rate: 1.0 mL/minute
 RF Power: 1300 watts
 Wavelength: Arsenic: 200.334 nm; Confirmatory: 188.979 nm
 Data Acquisition: Win Lab

Microwave Digester Conditions:

Instrument: Milestone Ethos EZ

Time (minutes)	Temperature (°C)	Power (Watts)
0-15	0-100	1200
15-35	100	1200
35-55	Cooling period	0

A summary of the validation data are given in Table 2.

Table 2 Summary of method validation for method validated in study DNA4143 for the determination of arsenic content in elemental iron technical material

Matrix	Analyte	LOQ (g/kg) [†]	Impurity level prior to fortification (g/kg)	Recovery fortification level (g/kg)	Recoveries (%) (mean, n)	Repeatability % RSD (n)	Linearity	Specificity
Elemental Iron Technical Material	Arsenic	0.03	<0.003	0.003 [§] 0.03	84.41 – 108.2 (96.44, 6) [§] 79.66 – 85.73 (82.15, 6)	2.882 (6) at 0.03 g/kg Modified Horwitz: 6.425 Horrat $H_r = 0.449$	0.15 – 5 mg/L (0.003 – 0.1 g/kg) (n=7x2) $r^2 = 0.9954$ Equation: $y = 0.001x + 0.0956$	The ICP-OES method is highly specific to the analyte. Samples are analysed using the elements characterised emission wavelength. No interference with the solvent blank or other impurities was observed.

[†] The experimental determination of the LOQ is not required. The LOQ has been set with consideration to all of the validation data provided including the lowest fortification level and the lowest point in the linearity.

[§] Results determined using reference standards rather than test batches or spiked test batches.

As arsenic was not detected above the LOQ in the test samples, precision was determined using samples of batch no. 'DNA4043/1' spiked with arsenic reference standard solution. Accuracy at the 0.03 g/kg fortification level was also determined using these spiked samples. Recoveries at 0.003 g/kg level were determined by analysis of the reference standard alone.

No significant interference is observed between the analytes and the active substance; the analytical method used is considered highly specific to the analyte of interest. A certified reference standard and library of elemental wavelengths confirmed the identity of arsenic and the highly specific nature of this method. A full consideration of the confirmation of identity is given in Volume 4, section C.1.2.2.

The Horrat value determined is below the acceptable limit (≤ 1). The linear range extends over an appropriate range considering the level of arsenic in the specification.

The experimental determination of the LOQ is not required. The LOQ has been set at the lowest fortification level with supporting precision and accuracy data. The linear range extends at least 20% below this level therefore it is an appropriate LOQ to use as the specification limit.

The applicant originally proposed an LOQ of 0.003 g/kg. However this LOQ is not fully supported, given that based on the validation data currently available, the linear range does not extend below 0.003 g/kg and the accuracy results at this fortification level were determined using reference standards rather than test batches or fortified test batches.

The method of analysis is considered fully validated in accordance with both SANCO/3030/99 rev. 4 and rev. 5.

Mercury

Sample preparation: The assay of mercury was performed using approximately 0.1 g of technical material. The mass of the technical material was accurately recorded, transferred to a digestion vessel and made up in 5 mL of nitric acid and 5 mL of hydrochloric acid. The samples were then digested in a microwave digester for 55 minutes and the 10 mL digested sample was transferred to a 100 mL volumetric flask and made to volume with deionised water. These samples were further diluted 1:2 and used for assay by injecting each solution once into the AFS (Atomic Fluorescence Spectroscopy) under the following conditions:

AFS Conditions:

Instrument:	PS Analytical Mercury Analyser
Gain:	100
Mode:	Ratio
Measurement mode:	Peak height
Delay:	20 seconds
Analysis:	40 seconds
Memory:	60 seconds
Pump 1 Speed:	100%
Pump 2 Speed:	100%
Data collection:	Millennium
Reductant:	2% w/v Tin (II) Chloride, 10% v/v hydrochloric acid in water
Blank:	5% nitric acid and 5% hydrochloric acid in deionised water

Microwave Digester Conditions:

Instrument: Milestone Ethos EZ

Time (minutes)	Temperature (°C)	Power (Watts)
0-15	0-100	1200
15-35	100	1200
35-55	Cooling period	0

The reductant is a solution of 2% w/v Tin (II) Chloride, 10% v/v hydrochloric acid in water. This is used as a reagent which is pumped through the AFS instrument. The technical material is prepared in an acid solution. The acid reacts with the mercury in the technical material and ionises it. The sample is then injected into the instrument where it reacts with the reductant to produce mercury in its ground state so it can be taken up as a vapour into the detector. The vapour is analysed using AFS to detect the mercury content.

A summary of the validation data are given in

Table 3.

Table 3 Summary of method validation for method validated in study DNA4143 for the determination of mercury content in elemental iron technical material

Matrix	Analyte	LOQ (g/kg)†	Impurity level prior to fortification (g/kg)	Recovery fortification level (g/kg)	Recoveries (%) (mean, n)	Repeatability % RSD (n)	Linearity	Specificity
Elemental Iron Technical Material	Mercury	0.0001	<0.0001	0.0001 0.0005	78.17 – 91.30 (85.36, 6) 75.42 – 86.47 (80.97, 6)	6.12 (6) at 0.0001 g/kg Modified Horwitz: 15.16 Horrat H_r = 0.404 6.425 (6) at 0.0005 g/kg Modified Horwitz: 11.899 Horrat H_r = 0.540	0.05 – 0.75 µg/L (0.0001 – 0.0015 g/kg) (n=5x2) r^2 = 0.9992 Equation: $y = 0.000417x + 0.009899$	The mercury analyser is specific to mercury analysis and cannot be configured to measure any other metal anion or cation. Samples are analysed by the elements characterised emission wavelength. No interference with the solvent blank or other impurity standards.

†The experimental determination of the LOQ is not required. The LOQ has been set with consideration to all of the validation data provided including the lowest fortification level and the lowest point in the linearity.

As mercury was not detected above the LOQ in the test samples, precision was determined using samples of batch no. 'DNA4043/1' spiked with the reported levels of mercury using a certified reference standard. Accuracy was also determined using samples of batch no. 'DNA4043/1' spiked with the reported levels of mercury using a certified reference standard.

No significant interference is observed between the analyte and the active substance; the analytical method used is considered highly specific to the analyte of interest. A certified reference standard and library of elemental wavelengths confirmed the identity of mercury and the highly specific nature of this method. A full consideration of the confirmation of identity is given in Volume 4, section C.1.2.2.

The Horrat value determined is below the acceptable limit (≤ 1).

The experimental determination of the LOQ is not required. The LOQ has been set at the lowest point on the linearity plot. There is sufficient confidence that the method is working at this level (0.0001 g/kg). However, as mercury is considered a relevant impurity, the method should be validated at levels at least 20% lower than the specification; the linear range should extend at least 20% below.

The method of analysis is not considered fully validated in accordance with both SANCO/3030/99 rev. 4 and rev. 5 due to the method not being validated at a level at least 20% below the specification limit. However the method is considered fit for purpose and additional method validation data to support the proposed specification level of max. 0.0001 g/kg is set as a data gap.

Nickel and Cadmium

Sample preparation: The analysis was performed using approximately 2.5 g of technical material. The mass of the technical material was accurately recorded, transferred to a digestion vessel and made up in 5 mL of nitric acid and 5 mL of hydrochloric acid. The samples were then digested in a microwave digester for 55 minutes and the 10 mL digested sample was transferred to a 50 mL volumetric flask and made to volume with deionised water. These samples were used for assay by injecting each solution once into the ICP-OES under the following conditions:

ICP-OES Conditions:

Instrument: Perkin Elmer ICP-OES
 Mode: Axial
 Gas Flow: Plasma: 15 mL/minute; Auxiliary: 0.2 mL/minute; Nebulizer: 0.8 mL/minute
 Pump Flow Rate: 1.0 mL/minute
 RF Power: 1300 watts
 Data Acquisition: Win Lab
 Wavelength: Cadmium 228.802 nm; Nickel 231.604 nm

The samples were assayed by ICP-OES to determine the content of any elements greater than 0.5 g/kg in the technical material based on a multi-element standard containing cadmium and nickel.

Specific validation data to support this screening method have not been provided. However, the supporting ICP-OES data provided shows that the method is capable of detecting the range of metals present in the multi-element standard. Some further information is provided in Vol 4 C.1.5.1.2. Further validation data is required to support the validity of this method with regards to the two relevant impurities: cadmium and nickel. This has been identified as a data gap.

Report:	██████ G (2018) Analysis of Lead in Five Batches of Elemental Iron Powder. Report number: 18A11048-01-5B, Sponsor Reference number: R-39834
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Previous evaluation	None.
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Lead

Sample preparation: Approximately 500 mg of test item were accurately weighed into an Erlenmeyer flask. 20 mL of ultrapure water and 10 mL nitric acid (67%) were added. The flask was heated up on a heatable magnetic stirrer until a volume reduction to approximately 10 mL was achieved and white fumes appeared. A condensation bulb was settled on top of the flask and the solution boiled for another two hours. After cooling down, the solution was filled to 100 mL with ultrapure water in a volumetric flask. Final analysis was performed using GF-AAS (Graphite Furnace Atom Absorption Spectroscopy) under the following conditions:

GF-AAS Conditions:

Instrument: Graphite furnace AAS, Thermo Scientific ICE 3000 series
 Wavelength: Lead 283.3 nm (quantification); 217.0 nm (confirmation)
 Measurement time: 6 s
 Operation mode: Extinction
 Background correction: Zeeman
 Sample volume: 30 µL (20 µL sample, 10 µL matrix modifier)
 Program:

Temperature (°C)	Time elapsed (s)	Gradient (°C/s)	Gas flow (L/min)
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120	120	10	0.3
200	60	5	0.3
450	30	10	0.3
800	10	5	0.3
2300	6	0	Off
2500	3	50	0.3
20	15	0	0.3

A summary of the validation data are given in Table 4.

Table 4 Summary of method validation for method validated in study: 18A11048-01-5B for the determination of lead content in elemental iron technical material

Matrix	Analyte	LOQ (g/kg)†	Impurity level prior to fortification (g/kg)	Recovery fortification level (g/kg)	Recoveries (%) (mean, n)	Repeatability % RSD (n)	Linearity	Specificity
Elemental Iron Technical Material	Lead	0.003	<0.003	0.003 0.009	95.0 – 117.9 (103, 5) 113.3 – 130.5 (122, 5)	8.96 (5) at 0.003 g/kg Modified Horwitz: 9.086 Horrat $H_r = 0.986$ 5.60 (5) at 0.009 g/kg Modified Horwitz: 7.701 Horrat $H_r = 0.727$	3.5 – 35 $\mu\text{g/L}$ (0.0007 – 0.007 g/kg) (n = 7) Equation: $y = 0.0114x + 0.0254$	GF-AAS is a highly specific method using a specific hollow cathode lamp for lead. Independent quantification and confirmation wavelengths were analysed. No significant interference with the blank, active substance or other impurities were observed.

†The experimental determination of the LOQ is not required. The LOQ has been set with consideration to all of the validation data provided including the lowest fortification level and the lowest point in the linearity.

As lead was not detected above the LOQ in the test samples, precision was determined using samples of batch no. 'CIP Code 11048' (2290598) spiked with the reported levels of lead using a certified reference standard. Accuracy was also determined using samples of batch no. 'CIP Code 11048' (2290598) spiked with the reported levels of lead using a certified reference standard.

No significant interference is observed between the analyte and the active substance; the analytical method used is considered highly specific to the analyte of interest. Analysis of a certified reference standard and the test samples at two different wavelengths specific to lead confirmed the identity of lead and the highly specific nature of this method. A full consideration of the confirmation of identity is given in Volume 4, section C.1.2.2.

The Horrat value determined is below the acceptable limit (≤ 1). The linear range extends over an appropriate range considering the level of lead in the proposed specification.

The experimental determination of the LOQ is not required. The LOQ has been set at the lowest level at which acceptable accuracy and precision have been demonstrated. There is sufficient confidence that the method is working at this level (0.003 g/kg). Additionally, there is confidence that the method is working at levels 20% below this LOQ which is also the specification limit, as the linear range extends to a much lower level (0.0007 g/kg).

The method of analysis is considered fully validated in accordance with both SANCO/3030/99 rev. 4 and rev. 5.

Applicability of existing CIPAC Methods

A range of methods are available for the determination of arsenic, mercury and lead. They are all standard established methods which are based on specific chemistry and they are broadly accepted as appropriate for determination of heavy metals. In addition, the CIPAC methods MT 99 (arsenic), MT 92.1 (lead) and MT 110 (mercury) are available and they have been collaboratively tested.

The technical material is a material certified as “Food Grade Quality” according to Food Chemical Codex (FCC 2016) and is sold to be used as nutritional supplement. Following the FCC, only 3 relevant impurities, Arsenic, Lead and Mercury, have been specified in the specification.

The Food Chemicals Codex (FCC) provides a collection of internationally recognized standards for the purity and identity of food ingredients. Some methods are available for the relevant impurities. The FCC has been published since 1966. It provides essential criteria and analytical methods to authenticate and determine the quality of food ingredients.

These methods have not been considered further as fully validated methods for the determination of lead, mercury and arsenic content in the technical material has been reported (██████████ 2018, ██████████ 2018). These methods were reported by the applicant so have been reported below for completeness.

Report:	Anonymous (1981) Third Edition “Food Chemicals Codex”, page 151.
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Previous evaluation	None.
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Determination of arsenic content: The iron is dissolved in diluted sulfuric acid TS, heated on a steam bath until the evolution of hydrogen ceases, allowed to cool down before dilution with water. The determination of the Arsenic is done by Silver Diethyldithiocarbamate colorimetric method.

Iron ██████████: Dissolve 1 g in 25 mL of diluted sulfuric acid TS, and heat on a steam bath until the evolution of hydrogen ceases. Filter through a tared filter crucible, collecting the filtrate in a 100 mL volumetric flask, wash with water until free from sulfate, and dry at 105°C for 1 h. The weight of the residue does not exceed 12.5 mg. Dilute the filtrate to volume with water. Transfer 40 mL of the filtrate (equivalent to 400 mg of Fe) obtained into an arsine generator flask. Add 20 mL of dilute sulfuric acid (1 in 5), 2 mL of potassium iodide TS, and 0.5 mL of Stannous Chloride Solution, and mix. Allow the mixture to stand for 30 min at room temperature. Pack the scrubber tube with two plugs of Lead Acetate-Impregnated Cotton, leaving a small air space between the two plugs, lubricate joints b and d with stopcock grease, if necessary, and connect the scrubber unit with the absorber tube. Transfer 3.0 mL of Silver Diethyldithiocarbamate Solution to the absorber tube, add 3.0 g of granular zinc (20-mesh) to the mixture in the flask, and immediately insert the standard-taper joint in the flask. Allow the evolution of hydrogen and color development to proceed at room temperature (25 ± 3°C) for 45 min, swirling the flask gently at 10-min intervals. (The addition of a small amount of isopropanol to the generator flask may improve the uniformity of the rate of gas evolution.) Disconnect the absorber tube from the generator and scrubber units, and transfer the Silver Diethyldithiocarbamate Solution to a 1 cm absorption cell. Determine the absorbance at the wavelength of maximum absorption between 535 nm and 540 nm, with a suitable spectrophotometer or colorimeter, using Silver Diethyldithiocarbamate Solution as the blank. The absorbance due to any red color from the solution of the sample does not exceed that produced by 30 mL of Standard Arsenic Solution (3 µg As) when treated in the same manner and under the same conditions as the sample. The room temperature during the generation of arsine from the standard should be held to within ± 2°C of that observed during the determination of the sample.

Determination of lead content: Transfer 200 mg of the sample into a 150 mL beaker, and add 8 mL of hydrochloric acid and 2 mL of nitric acid. Prepare a control containing 5.0 mL of Diluted Standard Lead Solution (5 µg Pb), 8 mL of hydrochloric acid, and 2 mL of nitric acid, and carry the sample and the control solutions through the following procedure. After the initial reaction subsides, the sample is evaporated to dryness on a steam bath, allowed to cool down and dissolved in 10 mL diluted hydrochloric acid (1 in 2). 25 mL Ammonium Citrate Solution is added followed by a stronger ammonia TS (7 mL). After cooling down, the pH is adjusted to 9.0 using either stronger ammonia TS or hydrochloric acid and the sample is transferred to a separator. The sample is then extracted with 5 mL portions of Dithizone Extraction Solution until the extraction solution retains its original color and the extracts are combined in a second separator. The combined extracts are washed by shaking with Citrate Cyanide Wash Solution and the wash solution is washed with Dithizone Extraction Solution. The chloroform layers are combined, and 20 mL dilute nitric acid (1 in 100) is added. After shaking and separation of the layers, the chloroform layer is shaken with dilute nitric acid (5 mL). The acid washes are combined, and the pH is adjusted with diluted ammonia TS to 2.5. The solution is then transferred into a separator where pH 2.5 Buffer Solution is added. Dithizone-Carbon Tetrachloride Solution is added. The carbon tetrachloride layer is washed with pH 2.5 Wash Solution, then the carbon tetrachloride layer is discarded, and the aqueous layers is combined. Ammonia-Cyanide Solution is added, and the sample is extracted with Dithizone-Carbon Tetrachloride Solution until the carbon tetrachloride shows no further pink color. The combined extracts are washed with Ammonia-Cyanide Wash Solution. The stem of the separator is dried, and the carbon tetrachloride is drained through a plug of cotton to remove the last trace of water. The absorbances of both solutions are determined in 1-cm cells at 520 nm with a suitable spectrophotometer, using carbon tetrachloride as the blank. The absorbance of the sample solution should not exceed that of the control.

Determination of mercury content: Because mercuric dithizonate is light-sensitive, this procedure should be performed in subdued light. Transfer 1 g of the sample into a 250 mL beaker, add 20 mL of dilute nitric acid (1 in 2), and digest on a steam bath for about 45 min. Add 5 mL of dilute hydrochloric acid (1 in 3), and continue heating on the steam bath until the sample is dissolved. Cool to room temperature, and filter, if necessary, through a medium-porosity filter paper. Wash with a few mL of water, add 20 mL of Sodium Citrate Solution and 1 mL of Hydroxylamine Hydrochloride Solution to the filtrate, and adjust the pH to 1.8 with stronger ammonia TS. A control sample is prepared by treating Diluted Standard Mercury Solution in the same manner and with the same reagents. The control and the sample are transferred into separate separators and both solutions are treated as follows: extract with Dithizone Extraction Solution. Drain carefully, collecting the chloroform in another separator. If the chloroform does not show a pronounced green color due to excess reagent, add additional (5 mL) extraction solution. Continue the extraction with 5-mL portions, if necessary, collecting each successive extract in the second separator, until the final chloroform layer contains dithizone in marked excess. To the combined chloroform extracts add dilute hydrochloric acid, shake the mixture vigorously and discard the chloroform. Extract with chloroform, drain carefully and discard the chloroform. Add 0.05 M disodium EDTA and 6N acetic acid to the aqueous layer. Slowly add ammonia TS and cool the separator. Transfer the solution into a 150 mL beaker, adjust the pH to 1.8 with ammonia TS or dilute nitric acid, using a pH meter and return the solution to the separator. Add Dithizone Extraction solution and shake vigorously. Allow the layers to separate, insert a plug of cotton into the stem of the separator and collect the dithizone extract in a test tube. Determine the absorbance of each solution in 1 cm cells at 490 nm with a suitable spectrophotometer, using chloroform as the blank. The absorbance of the Sample solution does not exceed that of the control.

These methods have not been considered further as a fully validated method for the determination of lead, mercury and arsenic content in the technical material has been reported (██████ 2018, ██████ 2018).

There are no co-formulants which are defined as relevant for toxicity (environmental or human health) therefore no methods are required. No stabilizers or additives are included in the active substance as manufactured therefore no methods are required.

Data gaps

The data gaps identified for additional method validation data to support the determination of the relevant impurities mercury, nickel and cadmium in the technical material are listed in Vol 1, section 3.1.4.5. The lack of these data should not impact the approval of the active substance as there is sufficient evidence that these data gaps are not safety concerns. There is confidence that the levels of relevant impurities found in the technical material

are sufficiently low, only data to fully support the method LOQ is required. Additionally, the source of the active substance holds approval in the USA as a food-grade mineral supplement.

B.5.1.2. Methods for risk assessment

B.5.1.2.1. Methods in soil, water, sediment, air and any additional matrices used in support of environmental fate studies

No studies that require method validation have been considered for the environmental fate and behaviour section of the evaluation.

B.5.1.2.2. Methods in soil, water and any additional matrices used in support of efficacy studies.

No studies that require method validation have been considered for the efficacy section of the evaluation.

B.5.1.2.3. Methods in feed, body fluids and tissues, air and any additional matrices used in support of toxicology studies

No studies that require method validation have been considered for the toxicology section of the evaluation.

B.5.1.2.4. Methods in body fluids, air and any additional matrices used in support of operator, worker, resident and bystander exposure studies

No studies that require method validation have been considered for the operator, worker, resident and bystander exposure section of the evaluation.

B.5.1.2.5. Methods in or on plants, plant products, processed food commodities, food of plant and animal origin, feed and any additional matrices used in support of residues studies

No studies that require method validation have been considered for the residues section of the evaluation.

B.5.1.2.6. Methods in soil, water, sediment, feed and any additional matrices used in support of ecotoxicology studies

No studies that require method validation have been considered for the ecotoxicology section of the evaluation. Methods for the determination of the product are reported in Volume 3CP B5 of the DAR.

B.5.1.2.7. Methods in water, buffer solutions, organic solvents and any additional matrices used in the physical and chemical properties tests

No studies that require method validation have been considered for the physical and chemical properties section of the evaluation.

B.5.2. METHODS FOR POST-APPROVAL CONTROL AND MONITORING PURPOSES

The active substance, elemental iron, is a stable, non-volatile elemental atomic particle and is insoluble in water. As iron is a natural constituent of soils serving as essential nutrient in animal and plant physiology, relevant residues of iron in food of plant, animal origin, environmental compartments, air and body fluids and tissues, in exceedance of natural background, are not expected to occur.

Also, Iron (Fe), molybdenum (Mo), and tin (Sn) are considered micronutrient metals essential for body function. Most of the iron in our body is found in the blood as haemoglobin, which is a protein used to carry oxygen to the body's tissues.

The active substance is certified as "Food grade Quality" according to Food Chemical Codex (FCC 2016).

There are already numerous international peer validated methods and ring-tested methods available. Therefore, no monitoring methods are required.

B.5.2.1. Methods for the determination of all components included in the monitoring residue definition in or on food and feed of plant and animal origin

Relevant residues of elemental iron in food of plant and animal origin, in exceedance of natural background levels, are not expected to occur. Therefore no MRLs are proposed. Therefore, analytical methods for the determination of residues of the active substance or any metabolites in food of plant and animal origin for enforcement purposes are not required.

However, numerous international peer validated methods and ring-tested methods are available, e.g. Total iron is determined by almost all methods after acid digestion followed by element specific determination with AAS, ICP-OES or ICP-AES. This method principle has been successfully validated in numerous ring tested methods:

- ISO 5517:1978 (1978), Fruit, vegetables and processed commodities. Determination of iron content. Photometric method
- ISO 9526:1990 (1990), Fruit, vegetables and processed commodities. Determination of iron content by FAAS
- DIN EN 16943 (2017), Food products, determination of calcium, copper, iron, magnesium manganese, phosphorus, potassium, sodium, sulfur and zinc by ICP-OES
- DIN EN 14082 (2003), Food products, determination of lead, cadmium, zinc, copper, iron and chromium by AAS after dry calcination
- DIN EN 14084 (2003), Food products, determination of lead, cadmium, zinc, copper and iron by AAS after digestion by microwave
- DIN EN ISO 6869 (2001), Animal feed, determination of calcium, copper, iron, magnesium, manganese, potassium, sodium and zinc by AAS
- DIN EN 15510 (2007), Animal feed, determination of calcium, sodium, phosphorus, magnesium, potassium, iron, zinc, copper, manganese, cobalt, molybdenum, arsenic, lead and cadmium by ICP-AES
- DIN EN 15621 (2012), Animal feed, determination of calcium, sodium, phosphorus, magnesium, potassium, sulfur, iron, zinc, copper, manganese and cobalt by ICP-AES after digestion under pressure
- DIN EN 8294 (1999), Fatty product from animal and vegetable origins, determination of copper, iron and nickel by AAS with graphite furnace.
- NF V05-126 (1982), Fruit, vegetables and processed commodities. Determination of iron content by FAAS
- NF V76-113 (1982), Fruit juices and vegetable juices. Determination of copper, iron and zinc content by FAAS
- DS EN 13805:2014 (2014), Foodstuffs. Determination of trace elements – pressure digestion

The following published methods were presented by the applicant with validation data for the determination of elemental iron in oily and dry matrices, animal tissue and feed. These methods have not been relied upon but reported for completeness.

Report:	García P., Romero C, Brenes M., Garrido A. (2002) Validation of a method for the analysis of iron and manganese in table olives by flame atomic absorption spectrometry. Report number: J Agric Food Chem. 2002 Jun 19;50(13):3654-9.
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Previous evaluation	None.
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The aim of this work was the evaluation and validation of a method, based on flame atomic absorption spectrometry, to be applied for Fe and Mn determination in ripe olives and table olives in general.

Sample preparation:

Samples were of Hojiblanca, Manzanilla, and Cacereña cultivars, processed as ripe olives at both pilot and industrial scales.

The pulp of 100 g of brined olives was separated from the pit by manual or automatic pitting machine, ground, and homogenized. From the resulting paste, 2 g was exactly weighted in a quartz capsule containing 1 mL of magnesium nitrate solution, which has the double function of facilitating the combustion; the small ashes were retained and placed in a capsule, avoiding possible losses due to the adsorption of the vessel walls. The capsule was put over an electrical heating plate and the temperature increased gradually to 350°C. The capsule was then put in a muffle oven and incinerated at 550°C. To this end the temperature was quickly brought to 250°C and then increased slowly until the calcination temperature had been reached, which was maintained for ~8-10 h.

The ashes, white-grayish in color, were slightly moistened and dissolved with three parts of 2 mL of 6N hydrochloric acid and filtered, part by part, through a filter paper into a 25-mL volumetric flask. After that, the filter was cleaned three times with 3 mL of deionized water, which was also added to the volumetric flask, and it was completed with deionized water until level. The dissolution can be aided by heating slightly the capsule in every addition of hydrochloric acid. To make it easier, the filtration can be made by means of a suction hood. At the same time, a blank was prepared with only the reagents.

AAS conditions:

Instrument:	A GBC model 932 AA (Victoria, Australia) atomic absorption spectrometer equipped with two hollow multielement cathode lamps, (Cu, Cr, Co, Fe, Mn, and Ni)
Lamp current (mA):	10 (Fe) 5.0 (Mn)
Wavelength (nm):	248.3 (Fe) 279.8 (Mn)
Band-pass (nm):	0.2
Flame type:	Oxidizing (Fe) Stoichiometric (Mn)
Instrument mode:	Absorbance
Optimum working range:	2.0-9.0 mg/L (Fe) 1.0 – 3.6 mg/L (Mn)

Summary of validation data:

Specificity: Table olives are rich in several mineral elements, Na, Ca, and K being the most important. There was no significant effect due to the matrix, but a slight bias due to the presence of Ca has been detected. Thus, it is advisable that calibration curves for Fe determination be prepared from Fe (or Fe and Mn) standard solutions containing at least 20 mg/L of Ca to prevent this systematic constant slight error due to the usual presence of Ca in all of the commercial table olive samples and their ashes.

Linearity: Typical situation Lambert-Beer's law:

$$\text{Absorbance} = \log[I_0/(I_t + K)],$$

where I_t is the absorbable radiation

K is the nonabsorbable component

In the range of concentrations used in this study (2 mg/L to 12.48 mg/L), the plot of absorbance versus Fe or Mn concentrations was never a straight line. This situation is usual because there is always a part of the radiation that cannot be absorbed, because either it is an unresolved non-absorbing line adjacent to the absorbing line, the light path does not completely go through the flame, it strays or is scattered.

Accuracy and Precision: There is no certified material for Fe and Mn content in table olives. Trueness was judged by recovery experiments. The results are given in Table 5 and Table 6.

Table 5 Average recovery of Fe added for different samples (matrices) of ripe table olives

	Fe recovery for amount of Fe added		
Matrix	20.20 mg/kg	60.60 mg/kg	101.00 mg/kg
1	100.1% (n=3)	99.8% (n=3)	99.5% (n=3)
2	98.7% (n=3)	99.1% (n=3)	99.4% (n=3)
3	99.0% (n=3)	99.9% (n=3)	99.7% (n=3)

Precision was evaluated in all of the experiments carried out in this work because practically all of them were carried out in triplicate. Relative errors were, in general, below 4% and repeatability was below 3.43 mg/kg of olive paste.

Table 6 Average recovery of Mn added for different samples (matrices) of ripe table olives

	Mn recovery for amount of Mn added		
Matrix	10.10 mg/kg	25.25 mg/kg	45.45 mg/kg
1	99.21% (n=3)	99.09% (n=3)	99.2% (n=3)
2	98.38% (n=3)	99.64% (n=3)	100.07% (n=3)
3	99.42% (n=3)	99.72% (n=3)	98.90% (n=3)

Relative errors were, in general, below 3% and repeatability was below 0.38 mg/kg of olive paste.

Limit of Detection and Limit of Quantification: The method has detection limits of 0.106 mg/L for Fe and 0.022 mg/L for Mn and quantification limits of 0.271 mg/L for Fe and 0.057 mg/L for Mn, referred to the solution to be measured.

A consideration of the validity of this method in the context of the SANCO/825/00 rev. 8.1 guidance has not been made as monitoring methods for food and feed of plant and animal origin are not considered necessary for elemental iron. This method provided by the applicant has been reported for completeness.

Report:	Bou R., Guardiola F., Padró A., Pelfort E., Codony R. (2002) Validation of mineralisation procedures for the determination of selenium, zinc, iron and copper in chicken meat and feed samples by ICP-AES and ICP-MS. Report number: J. Anal. At. Spectrom., 2004, 19, 1361-13691
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Previous evaluation	None.
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Mineralisation procedures for determining Fe, Zn, Cu and Se in chicken meat and feed samples were studied. We employed three different sample preparation procedures to determine these elements by ICP-AES and ICP-MS. A closed vessel microwave mineralisation procedure was developed for chicken meat samples.

ICP-AES Conditions:

Instrument:	Optima 3200RL model ICP-AES (PerkinElmer, Norwalk, CT) (Fe, Zn, Cu determination)
Sample introduction:	"Cross flow" nebulizer
Auxiliary Ar flow:	0.5 L/min
Sample flow:	1 mL/min
Nebulizer gas flow:	0.8 L/min
Plasma	
Alumina injector	
Frequency:	40 MHz
RF power:	1150 W
Plasma gas flow:	15 L/min
Measures	
Background:	-0.04 nm
Plasma height:	15 mm
Read delay:	15 s
Integration time:	2-5 s
Replicates:	3
Emission lines:	Fe: 259.939 and 238.204 nm, Zn: 213.857 and 206.200 nm, Cu: 324.752 and 327.393 nm

Samples:

Five samples of mixed raw chicken meat, including its skin and two feed samples, were used to optimise the mineralisation procedure.

Digestion procedure (using closed PTFE vessels and microwave oven)

Procedure IIIa: Feed samples (0.25 g, wet weight) were accurately weighed into digestion PTFE vessels. Two procedures were compared. In Procedure IIIa, 5 mL of nitric acid, 2 mL of hydrogen peroxide and 1 mL of Milli-Q water were added to each tube which was then closed. Digestion was conducted by applying a 4-step program described as follows: firstly, heating at a rate of 10°C/min up to 120°C and holding for 5 min, then at 10°C/min up to 150°C and holding for 5 min, again at 10°C/min up to 180°C and a 5 min hold, and finally at 10°C/min up to 200°C with holding for 10 min. After samples had been allowed to cool, the digested solutions were transferred into 25 mL volumetric flasks and diluted to volume with Milli-Q water.

Procedure IIIb: By contrast, another procedure was carried out as follows: 4 mL of nitric acid, 2 mL of hydrogen peroxide and 2 mL of Milli-Q water were added to each tube, which was next closed. Digestion was completed according to the following program: heating at a rate of 5°C/min up to 100°C and hold 5 min, then at 10°C/min up to 180°C and hold for 5 min, then at 10°C/min up to 200°C and hold for 5 min, and finally at 10°C/min up to 210°C and hold for 10 min. After the samples had been allowed to cool, 1 mL of hydrofluoric acid was added and the samples were heated at a rate of 10°C/min up to 120°C and held 10 min. After the samples had cooled, 20 mL

of MilliQ water were added and these solutions were weighed. Three 1 mL aliquots of each mineralised solution were also weighed to determine the density, which was used for the calculation of the final solution volume.

Digested samples were diluted to an adequate element concentration and final acid matrix (approximately 2% or above) and were analysed using the ICP-AES. Two wavelengths were measured for each element (238.204 and 259.939 nm, 213.857 and 206.200 nm, 324.752 and 327.393 for Fe, Zn and Cu, respectively). The selected wavelengths were 259.939 nm for Fe, 213.857 nm for Zn, and 324.752 for Cu.

Validation data (Procedure IIIa and IIIb):

Linearity: Aqueous (1-2% HNO₃) calibration curves (intercept equal to 0) were used for quantification and the selected wavelengths were 259.939 nm for Fe.

Accuracy: Recoveries in spiked chicken meat were 101–103% for procedure IIIb (shown in Table 7).

Table 7 Recoveries of Fe in spiked chicken meat

Fortification level (mg/kg)	0	170	340	680
Recovery (mg/kg)	175 ± 7.7	352 ± 12	516 ± 17	863 ± 35
Recovery (%)	4.4	3.5	3.2	4.1
RSD (%)	-	103	101	103
n	8	8	8	8

Precision: The respective precisions of the element determinations after mineralisation by Procedures IIIa and IIIb were compared by analysing 8 aliquots of a feed sample. Aliquots of another feed sample (supplemented with organic selenium from Se enriched yeast) were analysed following Procedure IIIb to assess the element recoveries at different concentration levels. The different concentration levels were obtained by adding 0.5 mL of aqueous solution prepared with different amounts of the standard solutions of Zn, Fe, Se and Cu purchased from SCP Science. A summary is given in

Table 8 Summary of precision data for Fe in chicken meat

Element	Procedure IIIa HNO ₃ -H ₂ O ₂		Procedure IIIb HNO ₃ -H ₂ O ₂ -HF	
	Concentration (mg/kg) mean ± SD	RSD (%)	Concentration (mg/kg) mean ± SD	RSD (%)
Fe	272 ± 4.4	3.9	280 ± 8.2	1.8

The results obtained for iron using the procedure IIIa presented a much lower variability (2.0% of RSD) and were consistent with the certified values for the reference material being assessed.

The method proposed (procedure IIIb) describes a rapid and suitable mineralisation procedure in closed vessels for iron determination in feeds.

A consideration of the validity of this method in the context of the SANCO/825/00 rev. 8.1 guidance has not been made as monitoring methods for food and feed of plant and animal origin are not considered necessary for elemental iron. This method provided by the applicant has been reported for completeness.

Report:	Sanchez-Viffas M., M. Bagur G., Gazquez D., Camino M., Romero R., (1999) Determination of Tin, Vanadium, Iron, and Molybdenum in Various Matrices by Atomic Absorption Spectrometry Using a Simultaneous Liquid-Liquid Extraction Procedure. Report number: Journal of Analytical Toxicology, Vol. 23, March/April 1999
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Previous evaluation	None.
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Validation of an atomic-absorption spectrometric method for the determination of elemental iron in two certified reference materials, food samples, and petroleum crude. After treatment with acids, these elements are separated from matrix elements by simultaneous solvent extraction of 5,5'-methylenedisalicylohydroxamic acid complexes from HCl/NaClO₄ solution into an isobutyl methyl ketone/tributyl phosphate solution.

Sample preparation:

The food samples (oyster tissue, spanish lentils, rice, and clam tissue) and the sediment sample were dried at 110°C until they were of constant weight and ground into a fine powder, if necessary. The appropriate quantity of each sample was treated with HNO₃ in a sand bath (approximately 25 mL of acid for 5 g of sample), adding more nitric acid as necessary until foaming ceased. Ten milliliters of HClO₄ (70%, w/w) was then added, and the solution was evaporated to dryness. This process was performed twice. The residue was treated with 0.25M HCl solution to boiling, cooled, and filtered (Whatman no. 40 paper) and the solution was finally diluted to a known volume.

AAS conditions:

Instrument: Perkin-Elmer (Norwalk, CT) model 2380 atomic absorption spectrophotometer (AAS)
 Wavelength: 248.3 nm
 Lamp current: 30 mA
 Slit width: 0.2 nm
 Flame: air/acetylene

Validation data:

Linearity: triplicate responses at each concentration. A summary is given in Table 9.

Table 9 Summary of linearity data

Linearity range	Linearity	Sensitivity	RSD
0.06 – 0.50 µg/mL	99.0%	0.008 µg/mL	1.2%

Limit of detection: 0.012 µg/mL (n=10)

Specificity: detailed study of interferences was made for cations and anions in amounts ranging up to 50 mg with 12.5 µg of Fe. Ions were not considered as interfering if the mean value of three replicates produced an error in absorbance of less than 5%. There was no interference.

Accuracy and precision: To check the applicability of the proposed method, the following samples, which contained two or more of the four metal ions, were analyzed:

- two reference material (RM) samples: oyster tissue (SRM 1566) and Buffalo River sediment (CRM, N27-04), and
- five noncertified materials, a petroleum crude for Fe and V, a Spanish lentil sample for Fe and Mo and a clam tissue for Fe and V, and two different rice samples that were spiked with V and Mo (rice 1) and Sn (rice 2) because they only contained iron.

Accuracy data are summarised in Table 10.

Table 10 Summary of accuracy and precision data

Matrix	Other method # or certified value * (µg/g)	Found value (µg/g)	RSD (%)	n
Oyster tissue (SRM 1566)	195*	194	1.24	6
Buffalo river sediment (CRM, N27-04)	4.11*	4.2	3.8	6
Spanish lentils	62.12 [#]	61	5.42	7
Clam tissue	500 [#]	497	3.99	6
Light Arabia	1.69 [#]	1.75	3.90	4
Rice 1	5.93 [#]	6.0	9.00	5
Rice 2	45.1 [#]	44.6	0.73	3

* Certified values

[#] Determined by spectrometry using bathofenanthroline disulfonic acid disodium salt

The method is reliable, at least, for the samples analyzed. This is confirmed by the good agreement between the results and the certified values, or those obtained by other methods, and also by the recovery values (rice samples). Likewise, the RSD values indicate a satisfactory precision. Matrix effect: no matrix effect was observed for all matrix.

The procedures of acid digestion and determination by AAS or ICP-OES are generally recognised standard methods of analysis for inorganic elements such as iron.

A consideration of the validity of this method in the context of the SANCO/825/00 rev. 8.1 guidance has not been made as monitoring methods for food and feed of plant and animal origin are not considered necessary for elemental iron. This method provided by the applicant has been reported for completeness.

B.5.2.2. Methods for the determination of all components included for monitoring purposes in the residue definitions for soil and water

The active substance, elemental iron, is a stable, non-volatile elemental atomic particle and therefore cannot be degraded. Soil application of the plant protection product will not result in relevant additional residues of elemental iron in environmental compartments compared to natural background. Therefore, no monitoring methods for the determination of elemental iron in environmental compartments are required.

However, numerous international peer validated methods and ring-tested methods are available, e.g.

- ISO/TS 16965:2013 (2013), Soil Quality, Determination of iron content by ICP-MS
- DIN 19682 (2009), Soil Quality, Determination of carbonates, sulfides, pH and Fe²⁺ ions
- ISO 6332:1988 (1988), Water Quality, Determination of iron content. Spectrometric method using 1,10-phenanthroline, LOQ 0.01 mg/L
- DIN 38406 (2000), Normalised German Methods for analysis of iron in water, surface water and sludge. Determination of iron by AAS
- ASTM Designation: D 1068-0, Standard Test Methods for Iron in Water
- NEMI Standard Methods: 3500-Fe B, Iron by Phenanthroline (water)
- BS EN ISO 11885:2009 (2009), Water Quality. Determination of selected elements by ICPOES

The following published methods were presented by the applicant with validation data for the determination of elemental iron in sediment and environmental samples. These methods have not been relied upon but reported for completeness.

Report:	Sanchez-Viffas M., M. Bagur G., Gazquez D., Camino M., Romero R., (1999) Determination of Tin, Vanadium, Iron, and Molybdenum in Various Matrices by Atomic Absorption Spectrometry Using a Simultaneous Liquid-Liquid Extraction Procedure. Report number: Journal of Analytical Toxicology, Vol. 23, March/April 1999
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Previous evaluation	None.
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Validation of an atomic-absorption spectrometric method for the determination of elemental iron in two certified reference materials, food samples, and petroleum crude. After treatment with acids, these elements are separated from matrix elements by simultaneous solvent extraction of 5,5'-methylenedisalicylohydroxamic acid complexes from HCl/NaClO₄ solution into an isobutyl methyl ketone/tributyl phosphate solution. The procedures of acid digestion and determination by AAS or ICP-OES are generally recognised standard methods of analysis for inorganic elements such as iron.

Details of this method and supporting validation data are reported under B.5.2.1 above.

A consideration of the validity of this method in the context of the SANCO/825/00 rev. 8.1 guidance has not been made as monitoring methods for soil and water are not considered necessary for elemental iron. This method provided by the applicant has been reported for completeness.

Report:	Rüdel H., Kösters J, Schörmann J. (2007) Determination of the Elemental Content of Environment Samples using ICP-OES. Report number: Umweltprobenbank des Bundes, July 2007
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Previous evaluation	None.
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This guideline describes the procedure for examining digestions of environmental samples by means of ICP-OES. This is a guideline, not a validation report. However, some validation data are given in the document.

Sample preparation: Digestion solutions are analysed under the same conditions as the analytical standards. If necessary, samples are diluted in order to be analysed within the working range of the method.

ICP-OES Conditions:

Instrument: Optical emission spectrometers with inductively coupled plasma (ICP-OES)
Wavelength: 259.94 nm
Reference materials: Spruce needles (CRM 101, BCR); Poplar leaves (NCS DC 73350, Institute of Geophysical and Geochemical Exploration, Langfang China); Pine needles (NIST 1575a, NIST)

Validation data:

Limit of detection: 0.9 – 1.2 µg/g

Linearity: standard addition test is carried out. Method is linear but no additional data is provided.

Specificity: No interference was observed at the wavelength of 259.94 nm.

Accuracy/Precision: When analyzing samples for the Environmental Specimen Bank, certified reference materials are also analysed to detect any problems caused by incomplete digestion or losses. The reproducibility is calculated from the correspondence data of the reference materials via the relative standard deviation (where $n \geq 5$). A summary is given in Table 11 and Table 12.

Table 11 Summary of the analysis of certified materials (iron only)

Reference material	Certified content	Recovery (%)	SD (%)	n	Comment
Pine needles (NIST 1575 a)	46.0 µg/g	99.8	6.8	2	Certified value
Spruce needles (BCR CRM 101)	151 µg/g	99.7	3.1	5	Certified value
Beech leaves (BCR CRM 100)	535 µg/g	95.2	1.7	8	Certified value
Poplar leaves (NCS DC73350)	274 µg/g	86.6	0.9	4	Certified value
Sea lettuce (BCR CRM 279)	2300 µg/g	96.7	2.7	5	Informative value

Table 12 Summary of the analysis of reference materials from the Environmental Specimen Bank (IS UPS – Information system of the Environmental Specimen Bank; iron only)

Reference material	Concentration (aus IS-UPB)	Recovery (%)	SD (%)	n
Pine shoots (Dübener Heide 1992)	277 µg/g	89.7	1.5	2
Beech leaves (Hochharz 1996)	81.0 µg/g	95.0	1.3	7
Bladder wrack (Eckwarderhölme)	489 µg/g	88.2	0.6	4

The procedures of acid digestion and determination by AAS or ICP-OES are generally recognised standard methods of analysis for inorganic elements such as iron.

A consideration of the validity of this method in the context of the SANCO/825/00 rev. 8.1 guidance has not been made as monitoring methods for soil and water are not considered necessary for elemental iron. This method provided by the applicant has been reported for completeness.

B.5.2.3. Methods for the analysis in air of the active substance and relevant breakdown products formed during or after application

Methods for the determination of residues in air are not required since the active substance is certified as “Food grade Quality” according to Food Chemical Codex (FCC 2016). Additionally, iron is not classified as very toxic, toxic, harmful or irritant (T, T+, Xn, Xi). The active substance, elemental iron, is also a non-volatile elemental atomic particle and therefore relevant residues of elemental iron in air are not expected to occur. Therefore, no monitoring methods for the determination of elemental iron in air are required.

B.5.2.4. Methods for the analysis in body fluids and tissues for the active substance and relevant metabolites

Iron (Fe) is considered as a metal of minimal safety concern. It has a well established safety profile, no significant toxicity, is well tolerated, and is typically ubiquitous in nature. Also, the active substance, elemental iron, is certified as “Food grade Quality” according to Food Chemical Codex (FCC 2016). Relevant residues of elemental iron in body fluids and tissues distinguishable from the naturally occurring levels are not expected. Therefore, methods for the determination of elemental iron residues in body fluids and tissues are not required.

However, there are some publically available publications which provide some validation data for the determination of elemental iron in tissue and human plasma. The following published methods were presented by the applicant with validation data for the determination of elemental iron in tissue and human plasma. These methods have not been relied upon but reported for completeness.

Report:	Bou R., Guardiola F., Padró A., Pelfort E., Codony R. (2002) Validation of mineralisation procedures for the determination of selenium, zinc, iron and copper in chicken meat and feed samples by ICP-AES and ICP-MS. Report number: J. Anal. At. Spectrom., 2004, 19, 1361-13691
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Previous evaluation	None.
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Mineralisation procedures for determining Fe, Zn, Cu and Se in chicken meat and feed samples were studied. We employed three different sample preparation procedures to determine these elements by ICP-AES and ICP-MS. A closed vessel microwave mineralisation procedure was developed for chicken meat samples.

The procedures of acid digestion and determination by AAS or ICP-OES are generally recognised standard methods of analysis for inorganic elements such as iron.

Details of this method and supporting validation data are reported under B.5.2.1 above.

A consideration of the validity of this method in the context of the SANCO/825/00 rev. 8.1 guidance has not been made as monitoring methods for body fluids and tissues are not considered necessary for elemental iron. This method provided by the applicant has been reported for completeness.

Report:	Whitmire M., Osredkar A., Ammerman J., Biss J, Huang C., Marshall T., Ehringer K., de Lizio P. (2011) Full Validation of a High Resolution ICP-MS Bioanalysis Method for Elemental iron in Human Plasma with K₂EDTA. Report number: Whitmire et al., J Chromatograph Separat Techniq 2011, S4
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Previous evaluation	None.
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Inductively coupled plasma mass spectrometry (ICP-MS) has been used to provide accurate, sensitive, specific quantification of heavy metals in environmental samples, forensic samples, nutraceutical products, and drug articles (drug substances, drug products, and excipients). The main advantages of ICP-MS over the classical methods are its ability to accurately quantify trace metals at much lower detection limits, analytical speed, relative lack of interference, and definitive multiple isotope capability.

This report contains validation for a bioanalytical inductively coupled plasma mass spectrometry (ICP-MS) method for quantification of elemental iron in human plasma with K₂EDTA (matrix). This method was validated using typical bioanalytical validation parameters. The method was designed for quantitation of a drug substance containing an elemental iron core after administration to human patients, but it may be applicable to any analysis for elemental iron in human plasma, no matter the origin.

Materials and methods:

Elemental iron reference standards and germanium internal standard (IS) were from Ultra Scientific. Reagent water had a resistivity $\geq 18 \text{ M}\Omega\text{cm}$. Nitric acid and hydrochloric acid were ultrapure grade. Blank matrix was purchased from BioReclamation.

Centrifuge tube pre-treatment and pre-screening:

All 15 mL centrifuge tubes were pre-treated and pre-screened before use. All tubes and caps were soaked for a minimum of 12 hours with a 1% HNO₃ in water wash solution in a covered container. The tubes and caps were rinsed with reagent water with a resistivity $\geq 18 \text{ M}\Omega\text{cm}$. Tubes and caps were racked and allowed to air dry for prescreening. Approximately 10 mL of diluent was added to each pretreated tube, and tubes were capped and mixed on a Roto-shake Genie at the maximum speed for 2 minutes. The diluent from each tube was then analyzed by HR ICP-MS, along with the first standard curve sample. The acceptance criteria was $\leq 20\%$ of the total elemental iron response in the first standard curve sample. Tubes that failed this limit were discarded. Tubes that met this limit were rinsed with reagent water, racked, and allowed to air dry.

Extraction procedure:

1. Matrix was thawed unassisted at ambient temperature.
2. Matrix samples were mixed by vortexing.
3. 100 μL aliquots of QC control, QC sample, or calibration standard were added to pre-screened 15.0 mL centrifuge tubes.
4. 100 μL aliquots of Internal Standard Working Solution (ISWS) were added to all tubes except the double blank tubes.
5. 100 μL aliquots of diluent were added to the double blank tubes.
6. 9.80 mL aliquots of diluent were added to each tube. Tubes were capped and the contents mixed thoroughly by inversion on the Roto-shake Genie at the maximum speed for 2 minutes.
7. Samples were analyzed by HR ICP-MS.

Instrument:	Thermo-Finnigan ELEMENT2 High Resolution ICP-MS
RF power:	1.3 kW
Ar cool gas flow:	16 L/min
Ar plasma gas flow:	0.80 L/min
Ar nebulizer gas flow:	0.8 – 1.1 L/min
Nebulizer:	PFA-100 micro flow
Spray chamber:	Cyclonic
Detector mode:	Dual
Units:	cps
Peristaltic pump speed:	12 R/min
Pump tubing:	Black-black (0.76 mm i.d. x 150 mm 2-stop, PVC)
Sample uptake time:	120 s
Wash time:	120 s
Resolution mode:	Medium
Sample time:	20 ms
Samples/peak:	30
Mass:	Fe m/z 57, Ge m/z 72

Matrix: Eight lots of matrix were evaluated for selectivity. Among these lots, the following special considerations were included: at least one lot of lipemic plasma, at least one lot of haemolytic plasma, at least one plasma lot

from a male over age 60 with Type II Diabetes, and at least three lots of plasma from males and three from females. None of the eight lots of matrix tested for selectivity had a significant response for the Internal Standard (IS) (IS peak response in plasma blanks $\leq 5.0\%$ of the mean IS response in the LLOQ standards). Upon final testing, all analyzed matrix types successfully met the acceptance criteria for mean accuracy within $\pm 20.0\%$ RE of the nominal concentration when fortified at the LLOQ concentration. Therefore, the method was selective for elemental iron and the IS. However, precaution should be taken with sample collection to not lyse the red blood cells, thus releasing more elemental iron into the plasma and elevating the total elemental iron content above typical endogenous levels.

Validation data:

- Linearity was assessed using eight standards prepared in proxy matrix over a range equivalent to 0.5 -20 μg elemental iron/mL of matrix at the beginning and end of each run. Linearity was consistently demonstrated. Typical calibration was $y = 0.0659x + 0.0027$, with $R^2 = 0.999$.
- Quality control (QC) samples were prepared by fortifying matrix at 0.5, 1.5, 10, 15 and 100 $\mu\text{g/mL}$. A blank containing IS and a double blank were included in every run. Recoveries for all calibration standards and QCs were within the acceptance criteria.
- Ruggedness was analyzed in one data set ($n=6$ at each concentration) using a different preparation analyst from the three core validation runs. The acceptance criteria were a mean concentration within $\pm 15.0\%$ RE of the nominal ($\leq 20.0\%$ for LLOQ) and $\leq 15.0\%$ RSD ($\leq 20.0\%$ for LLOQ). The average RE for the LLOQ samples was 5.2%, and the RSD was 5.2%. The average RE for the QC Low samples was -0.7%, and the RSD was 4.6%. The average RE for the QC Mid samples was -0.8%, and the RSD was 2.2%. The average RE for the QC High samples was -1.9%, and the RSD was 3.6%. All acceptance criteria were met; therefore, the ruggedness of the assay was demonstrated.
- Limit of quantification: 0.5 $\mu\text{g/mL}$
- Selectivity was evaluated using six different lots of matrix fortified with IS and analyte at the LLOQ level.
- Storage stability: Freeze/thaw was demonstrated for 4 cycles. Bench top reproducibility was successful following 53 hours of ambient storage. ReInjection reproducibility was successful following 96 hours of ambient storage. Extract stability was successful following 190 hours of ambient storage. IS solution stability was successful following 29 days of ambient storage. Calibration standard stability was successful following 8 days of ambient storage. Analyte stock solution
- Stability was successful following 29 days of ambient storage. Long term storage stability was successful following 31 days at 2 to 8°C, 31 days at -15 to -25°C, and 30 days at -70 to -90°C storage.

System suitability, selectivity, carry over and ruggedness all met the acceptance criteria.

A consideration of the validity of this method in the context of the SANCO/825/00 rev. 8.1 guidance has not been made as monitoring methods for body fluids and tissues are not considered necessary for elemental iron. This method provided by the applicant has been reported for completeness.

REFERENCES RELIED ON

Data Point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
B5.1.1	██████	2018	Validation of the Method of Determination of the Active Ingredient and Specified Impurities in Iron Powder Technical Material, in Compliance with Good Laboratory Practice. Report number: DNA4143, Sponsor Reference number: R-39836 GLP: Yes Not published	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies.	ADAMA	N/A
B5.1.1	██████	2018	Analysis of Lead in Five Batches of Elemental Iron Powder. Report number: 18A11048-01-5B, Sponsor Reference number: R-39834 GLP: Yes Not published	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies.	ADAMA	N/A
B5.1.1	Anonymous	1981	Third Edition “Food Chemicals Codex” p. 151. N/A N/A GLP: No Published: Yes	N	N	-	Published literature	N/A