ANNEX B

Laminarin

B.5 Methods of analysis

B.5.1 Analytical methods for formulation analysis (Annex IIA 4.1; Annex IIIA 5.1)

B.5.1.1 Analytical methods for the determination of pure active substance in the active substance as manufactured (Annex IIA 4.1.1 and 4.1.3)

- Method ME-0211 A – Laminarin : measuring by ionic chromatography, amperometric detection (Duval, 2001)

GLP :
No GLP-compliance stated

Principle of the method :
The purity of technical Laminarin is determined by High Performance Ionic Chromatography (HPIC) of the glucose produced after total acid hydrolysis.
An equal volume of sample solution (1 mg/mL technical Laminarin in water) and of 1 M HCl is mixed and heated at 100°C for 3 h. After cooling at room temperature, the hydrolysed solution is diluted 1/20 with phosphate buffer (pH 6.86) and the glucose obtained is determined by HPIC (PA1 + PA1 guard) using a Dionex DX300 system equipped with a GP40 quaternary gradient pump and an ED40 electrochemical detector in integrated amperometry mode. Quantification by external standardization (use of Laminarin standard (Sigma) for correction).

Findings :
Specificity – interferences : demonstrated by confirmatory method (HPLC)
- HPIC-result : 86.96%
- HPLC-result : 86.76%

Linearity : linearity range : 0.1 – 30 mg/L (n = 10)
$r^2 = 0.9997$
y (area) = 7836.2x – 963.17

Accuracy : recovery = 96.37%

Repeatability : RSD = 1.30% (n = 3)

Limit of quantification (LOQ) : 1.5 g/kg

Conclusions :
HPIC-method ME-0211 A is suitable for the determination of Laminarin in technical Laminarin. This method was used for the 5-batch analysis study (ME-0211 A is identical to ME-0207 B, as far as Laminarin determination is concerned) and it will be used for the final purity control of technical Laminarin at the end of the industrial process.

- SEP/00-067 : Analysis of Laminarin in water samples after a hydrosolubility test (CEE A6) (Quintelas, 2001a)

GLP :
GLP-compliance stated

Principle of the method :
Laminarin is determined after acid hydrolysis of the polysaccharide into glucose. The glucose is quantified as its trimethylsilyl derivative by GC using FID. Quantification by external standardization; Laminarin is expressed as its monomeric unit glucose.
Equal volumes of 10x diluted Laminarin sample solution and of 1M HCl are mixed and heated at 100°C for 3 h. After cooling at room temperature, the hydrolysed solution is diluted 10x with water, after which 2 mL of this solution is mixed with an equal volume of L-rhamnose solution (5000 mg/L) and circa 25 mL of acetonitrile. After drying, the D-glucose and L-rhamnose mixture is dissolved in 10 mL of dry pyridine and derivatised using Sil A solution (made with 18 mL of dry pyridine, 2 mL of trimethylchlorosilane and 6 mL of hexamethyldisilazane). The trimethylsilyl derivatives of D-glucose and L-rhamnose are analysed as their anomeric forms by GC (SPB-35; 0.5 µm) using FID; the quantification of trimethylsilyl L-rhamnose being used as an internal reference to follow the reproducibility of the derivatisation step.

Findings :
Specificity – interferences : chromatograms of reference solution, spiked samples and samples are shown

Linearity : linearity range : 0.1 – 200 mg/L (n = 13)
$r^2 = 0.9998$
$C = 4.758 \times 10^{-5}$ S (area) – 0.2923

Accuracy :

Spiking concentration (g/L)	Recovery (%)
0.1	85
1.0	96
100	99

Repeatability : RSD = 2.3% (n = 5)
Limit of quantification (LOQ) : 0.1 g/L of Laminarin expressed as glucose
Conclusions :
The GC-FID method, which has been used in several physicochemical and ecotoxicological studies on the a.s. and the preparation, has been validated for that purpose.

- FC98005 : Determination of pure Laminarin – Global method (Cruz, 1998)
- Validation of the method of the dosage of pure Laminarin : Global method – GLP study (Trebert, 2002)
GLP :
GLP-compliance stated for the validation study
Principle of the method : ME-0248 (Trad)
The purity of technical Laminarin is determined by High Performance Ionic Chromatography (HPIC) with amperometric detection, by direct injection (i.e. without preliminary hydrolysis).
The technical Laminarin sample is diluted with MilliQ-water, after which Laminarin is determined by HPIC (PA1 column + PA1 guard column) using a Dionex system equipped with a GP40 quaternary gradient pump and an ED40 electrochemical detector in integrated amperometry mode. Quantification by external standardization (reference item : Laminarin (Sigma)).
Findings :
Specificity – interferences : typical chromatograms of blank, Laminarin standard, Laminarin sample and fortified Laminarin sample are shown, demonstrating specificity and absence of interferences
Linearity : linearity range : 10 – 50 mg/L (n = 5)
$r^2 = 0.9996$
y (area) = 2953325 x – 2176257
Accuracy : mean recovery = 99.99% (n = 3; RSD = 0.2%)
Repeatability : RSD = 1.27% (n = 5; mean content = 95.92%)
Limit of quantification (LOQ) : 10%
Conclusions :
Global HPIC-method ME-0248 (Trad) is suitable for determination of Laminarin content in technical Laminarin. It will be used to follow the purity of technical Laminarin during the industrial process, from the stage of extraction to the last stage of purification.

B.5.1.2 Analytical methods for the determination of significant and/or relevant impurities and additives in the active substance as manufactured (Annex IIA 4.1.2 and 4.1.3)

Methods for the determination of significant impurities 1-8 (content ≥ 1 g/kg) in technical Laminarin are described in Annex C, point C.1.2.4.1. Validation data for these methods are summarized in Table B.5.1.2-1.

None of the impurities present are of toxicological, ecotoxicological or environmental concern.
There are no additives in technical Laminarin.

Conclusions
The submitted methods are suitable for determination of significant impurities (content ≥ 1 g/kg) in technical Laminarin.

Table B.5.1.2-1 : Validation of methods for determination of significant impurities

Impurity N° (Method N°)	Specificity	Linearity*	Repeatability (% RSD)	Accuracy (% recovery)	LOQ (mg/kg)
1 (ME-0207 B)	demonstrated (confirmation by HPLC)	0.1-10 mg/L (n = 6) $r^2 = 0.9981$ $y = 243.7x + 20.5$	5.28 (n=4)	99.16	13.3 g/kg
2 (ME-0204 B)	demonstrated (confirmation by ICP)	2-10 mg/L (n = 5) $r^2 = 0.9999$ $y = -0.00040x^2 + 0.01708x + 0.00018$	1.05 (n = 5)	107.5	0.44
3 (ME-0204 B)	demonstrated (confirmation by ICP)	1-5 mg/L (n = 5) $r^2 = 0.9996$ $y = -0.0014x^2 + 0.0426x + 0.0013$	1.18 (n = 5)	98.0	1.20
4 (ME-0204 B)	demonstrated (confirmation by ICP)	2-10 mg/L (n = 5) $r^2 = 0.9987$ $y = 5.10^{-5}x^2 + 0.0097x - 0.0011$	3.88 (n = 5)	82.6	12.0
5 (ME-0204 B)	demonstrated (confirmation by ICP)	2-6 mg/L (n = 3) $r^2 = 0.9993$ $y = -0.0017x^2 + 0.0356x + 0.0007$	1.00 (n = 5)	105.3	1.20
6 (ME-0201 A)	internationally accepted method	not relevant for this type of method	1.91 (n = 5)	95.3	0.0095 g/kg
7 (ME-0205 B)	demonstrated (confirmation by ICP)	0.5-20 mg/L (n = 6) $r^2 = 0.9990$ $y = 304.7670x – 93.9857$	3.77 (n = 5)	99.8	100
8 (ME-0202 B)	internationally accepted method	not relevant for this type of method	1.08 (n = 5)	87.4	0.1690 g/kg

* for impurities 2-5 : calibration line equation is quadratic

B.5.1.3 Analytical methods for the determination of the active substance in plant protection products (Annex IIIA 5.1.1 and 5.1.3)

- VB001210 : Laminarin determination in the preparation PHYLIQ – Total hydrolysis method (Cruz, 1999)

GLP :

No GLP-compliance stated

Principle of the method :

Laminarin is determined by High pH Anion Exchange Chromatography (HPAEC) coupled with pulsed amperometric detection. After pre-treatment of the sample and total acid hydrolysis into glucose, Laminarin is quantified as its monomeric unit.

The PHYLIQ sample is first filtered on two successive ion exchange resins (resp. Maxiclean SAX anion exchanger and Maxiclean SCX cation exchanger) in order to eliminate all the ionic molecules present, after which the deionised solution is hydrolysed with 1M HCl at 100°C for 3 h. After cooling to room temperature, the hydrolysed solution is diluted 100x with phosphate buffer (pH 6.86) and the glucose obtained is determined by HPAEC (Carbopac PA1 column + Carbopac PA1 guard column, both packed with 10 µm ∅ polystyrene/divinylbenzene substrate agglomerated with 350 nm Microbead quaternary amine functionalized latex) using a Dionex DX300 system equipped with a GP40 quaternary gradient pump and an ED40 electrochemical detector in integrated amperometry mode. Quantification by external standardization (glucose and rhamnose).

Findings :

No validation data were presented ; only a typical chromatogram of analytical standards was shown.

Conclusions :

The HPAEC-method has not yet been validated and is thus not acceptable.

- VB001211 : Laminarin determination in the preparation PHYLIQ – Global method (Cruz, 2000)
- Validation of the method of the dosage of pure Laminarin in a formulation PHYLIQ : Global method – GLP study (Trebert & Cousin, 2002)

GLP :
GLP-compliance stated for the validation report
Principle of the method : ME-0250 (Trad)
Laminarin is determined by High Performance Ionic Chromatography (HPIC) with amperometric detection, after pre-treatment of the sample.
The PHYLIQ sample is diluted with MilliQ-water and filtered on two successive ion exchange resins (resp. Maxiclean SAX anion exchanger and Maxiclean SCX cation exchanger) in order to eliminate all the ionic molecules present, after which the deionised solution is analysed by HPIC (PA1 column + PA1 guard column) using a Dionex system equipped with a GP40 quaternary gradient pump and an ED40 electrochemical detector in integrated amperometry mode. Quantification by external standardization (reference item = Laminarin (Sigma)) .
Findings :
Specificity – interferences : typical chromatograms of blank, Laminarin standard, blank formulation and PHYLIQ sample are shown, demonstrating specificity and absence of interfering peaks
Linearity : linearity range : 10 – 60 mg/L (n = 6)
$r^2 = 0.9999$
y (area) = 661684 x + 761749
Accuracy : mean recovery = 93.34% (n = 3; RSD = 0.24%)
Repeatability : RSD = 1.56% (n = 7; mean content = 36.51 g/kg)
Conclusions :
Global HPIC-method ME-0250 (Trad) is suitable for determination of Laminarin content in PHYLIQ formulation.

- SEP/00-103 : Quantification of Laminarin in PHYLIQ after a MT46 CIPAC test (Quintelas, 2001b)

GLP :
GLP-compliance stated
Principle of the method :
The Laminarin content in PHYLIQ is determined after total acid hydrolysis of the polysaccharide into glucose. The glucose is quantified as its trimethylsilyl derivative by GC using FID. Quantification by external standardization; Laminarin is expressed as its monomeric unit glucose.
Equal volumes of 10x diluted PHYLIQ sample solution and of 1M HCl are mixed and heated at 100°C for 3 h. After cooling to room temperature, the hydrolysed solution is diluted 10x with water, after which 2 mL of this solution is mixed with an equal volume of L-rhamnose solution (5000 mg/L) and circa 25 mL of acetonitrile. After drying, the D-glucose and L-rhamnose mixture is dissolved in 10 mL of dry pyridine and derivatised using Sil A solution (made with 18 mL of dry pyridine, 2 mL of trimethylchlorosilane and 6 mL of hexamethyldisilazane). The trimethylsilyl derivatives of D-glucose and L-rhamnose are analysed as their anomeric forms by GC (SPB-35; 1.0 µm) using FID; the quantification of trimethylsilyl L-rhamnose being used as an internal reference to follow the reproducibility of the derivatisation step.
Findings :
Specificity – interferences : not addressed as such
chromatograms of reference solution and samples are shown
Linearity : linearity range : 0.1 – 20 mg/L (n = 11)
$r^2 = 0.9999$
$C = 3.831 \times 10^{-5}$ S (area) – 0.0082
Accuracy : not addressed
Repeatability : not addressed
Conclusions :
The GC-FID method has only been partially validated. It has nevertheless been used in several physicochemical (storage stability) and ecotoxicological studies on the a.s. and the preparation.

As we considered this method to be unsuitable for use in storage stability studies (see point B.2.2.15), the notifier agreed to reconduct both accelerated and shelf-life stability studies using the validated "global method".

B.5.1.4 Analytical methods for the determination of relevant impurities and formulants in plant protection products (Annex IIIA 5.1.2 and 5.1.3)

No methods have been submitted. No impurities or formulants of toxicological, ecotoxicological or environmental concern are present in the plant protection product.

B.5.2 Analytical methods (residue) for plants, plant products, foodstuffs of plant and animal origin, feedingstuffs (Annex IIA 4.2.1; Annex IIIA 5.2.1)

No methods have been submitted.
Conclusions
Relevant residues of Laminarin in food of plant and animal origin are not expected to occur. The setting of MRL's is not necessary and a residue relevant to MRL is not defined (cfr. point B.7). Therefore, residue analytical methods for the determination of the a.s. in food of plant and animal origin for enforcement purposes are not required.

B.5.3 Analytical methods (residue) for soil, water, air (Annex IIA 4.2.2 to 4.2.4; Annex IIIA 5.2.2 to 5.2.4)

No methods have been submitted.
Conclusions
Relevant residues of Laminarin in the environmental compartments are not expected to occur. A residue relevant to the environment is not defined (cfr. point B.8.9). Therefore, residue analytical methods for the determination of the a.s. in soil, water and air for monitoring purposes are not required.

B.5.4 Analytical methods (residue) for body fluids and tissues (Annex IIA 4.2.5; Annex IIIA 5.2.5)

No methods have been submitted.
Conclusions
Methods for the determination of residues in body fluids and tissues are not required since the a.s. is not classified as toxic or highly toxic.

B.5.5 Evaluation and assessment

B.5.5.1 Analytical methods for formulation analysis

Table B.5.5.1-1 : Summary of analytical methods for technical active substance and formulation analysis

Matrix	Analyte	Type of method	Validation	References
technical active substance	Laminarin (as glucose)	HPIC with amperometric detection (after total acid hydrolysis)	Full	ME-0211 A (Duval, 2001)
	Laminarin (as trimethylsilyl derivative of glucose)	GC with FID (after total acid hydrolysis and derivatisation)	Full	SEP/00-067 (Quintelas, 2001a)
	Laminarin	HPIC with amperometric detection	Full	FC98005 = ME-0248 (Trad) (Cruz, 1998) (Trebert, 2002)
	Impurity 1	HPIC with amperometric detection (after total acid hydrolysis)	Full	ME-0207 B (Portail, 2000a)
	Impurities 2-5	FAAS	Full	ME-0204 B (Delacourt, 2000)
	Impurity 6	Potentiometry	Full	ME-0201 A (Trebert, 2000a)
	Impurity 7	HPIC with conductivity detector	Full	ME-0205 B) (Portail, 2000b)
	Impurity 8	Titrimetry	Full	ME-0202 B (Trebert, 2000b)

Matrix	Analyte	Type of method	Validation	References
formulation (SL)	Laminarin (as glucose)	HPAEC with amperometric detection (after total acid hydrolysis)	None	VB001210 (Cruz, 1999)
	Laminarine	HPIC with amperometric detection	Full	VB001211 = ME-0250 (Trad) (Cruz, 2000) (Trebert & Cousin, 2002)
	Laminarin (as trimethylsilyl derivative of glucose)	GC with FID (after total acid hydrolysis and derivatisation)	Partial (specificity, accuracy, repeatability are missing)	SEP/00-103 (Quintelas, 2001b)

Evaluation
Validated methods are available for the determination of the purity and the significant impurities of the technical a.s. and for the determination of the a.s. content of the formulation (SL).

No CIPAC methods exist for this a.s.

B.5.5.2 Analytical methods for residue analysis

Relevant residues of Laminarin in food of plant and animal origin and in the environmental compartments are not expected to occur. The setting of MRL's is not necessary and no residue is defined, neither with relevance to MRL nor with relevance to the environment (cfr. point B.7; point B.8.9). Therefore, residue analytical methods for the determination of the a.s. in food of plant and animal origin for enforcement purposes, as well as in soil, water and air for monitoring purposes are not required.
Methods for the determination of residues in body fluids and tissues are not required since the a.s. is not classified as toxic or highly toxic.

B.5.6 References relied on

B.5.6.1 Analytical methods for the active substance

Annex Point / Reference number	Author(s)	Year	Title Testing facility, Report n°, GLP or GEP Status published or not	Data Protection Claimed Y/N	*Owner*
IIA 4.1/01	DUVAL P.	2001	Laminarin : measuring by ionic chromatography, amperometric detector SGS Laboratoire Crépin - Study N° ME-0211A Non-GLP, unpublished	Y	GOË
IIA 4.1/02 (location : IIA 2.6/01)	QUINTELAS G.	2001a	Analysis of Laminarin in water samples after a hydrosolubility test (EEC A6) SEPC – Study N°00-907005-008 GLP, unpublished	Y	GOË

Annex Point / Reference number	Author(s)	Year	Title Testing facility, Report n°, GLP or GEP Status published or not	Data Protection Claimed Y/N	*Owner*
IIA 4.1/03	CRUZ F.	1998	Determination of pure Laminarin : global method Laboratoires GOËMAR S.A. Study N° FC98005 Non-GLP, unpublished	Y	GOË
IIA 4.1/03d	TREBERT R.	2002	Validation of the method of the dosage of pure Laminarin : Global method – GLP study SGS Laboratoire Crépin – Report N° BPL 200107/1309 – rev.2 GLP, unpublished	Y	GOË
IIA 4.1/04	PORTAIL A.	2000a	Confidential information : *Please refer to Document J.* SGS Laboratoire Crépin -Study N° ME-0207B Non-GLP, unpublished	Y	GOË
IIA 4.1/05	DELACOURT H.M.	2000	Confidential information : *Please refer to Document J.* SGS Laboratoire Crépin -Study N° ME-0204B Non-GLP, unpublished	Y	GOË
IIA 4.1/06	TREBERT R.	2000a	Confidential information : *Please refer to Document J.* SGS Laboratoire Crépin -Study N° ME-0201A Non-GLP, unpublished	Y	GOË
IIA 4.1/07	PORTAIL A.	2000b	Confidential information : *Please refer to Document J.* SGS Laboratoire Crépin -Study N° ME-0205B Non-GLP, unpublished	Y	GOË
IIA 4.1/08	TREBERT R.	2000b	Confidential information : *Please refer to Document J.* SGS Laboratoire Crépin -Study N° ME-0202B Non-GLP, unpublished	Y	GOË

B.5.6.2 Analytical methods for the plant protection product

Annex Point / Reference Number	**Author(s)**	**Year**	**Title Testing facility, Report No., GLP or GEP Status published or not**	**Data Protection Claimed Y/N**	Owner
IIIA 5.1/01	CRUZ F.	1999	Laminarin - Determination in the preparation Phyliq - total hydrolysis method Laboratoires GOËMAR S.A. Report N° VB001210 Non-GLP, unpublished	Y	GOË
IIIA 5.1/02	CRUZ F.	2000	Laminarin - Determination in the preparation Phyliq - global method Laboratoires GOËMAR S.A. Report N° VB001211 Non-GLP, unpublished	Y	GOË
IIIA 5.1/02c	TREBERT R. COUSIN C.	2002	Validation of the method of the dosage of pure Laminarin in a formulation PHYLIQ : Global method – GLP study SGS Laboratoire Crépin – Report N° BPL 200110/0352 – rev.2 GLP, unpublished	Y	GOË
IIIA 5.1/03 (location : IIIA 2.7/01)	QUINTELAS G.	2001b	Quantification of Laminarin in Phyliq after a MT46 CIPAC test DEFITRACES Study No. SEPC 00-907005-034 GLP, unpublished	Y	GOË