Section A1 Applicant

Annex Point IIA1

The "Warfarin Task Force" is comprised of the following four applicant companies:

1.1.1	**Applicant (1)**	**Name:**	Spiess Urania Chemicals GmbH
		Address:	Heidenkampsweg 77; 20097 Hamburg; Germany
		Telephone:	+49-40-236520
		Fax number:	+49-40-23652265
		E-mail address:	Zimmer@spiess-urania.com
1.1.2	**Applicant (2)**	**Name:**	Killgerm Chemicals
		Address:	115 Wakefield Road; Ossett, West Yorkshire; UK
		Telephone:	+44-1924-277631
		Fax number:	+44-1924-264757
		E-mail address:	philip.dalgliesh@killgerm.com
1.1.3	**Applicant (3)**	**Name:**	Hentschke & Sawatzki KG
		Address:	Leinestraße 17, 24539 Neumünster, Germany
		Telephone:	+49-4321-98720
		Fax number:	+49-4321-987299
		E-mail address:	lubik@hentschke-sawatzki.de
1.1.4	**Applicant (4)**	**Name:**	Vetyl Chemie GmbH
		Address:	Gewerbegebiet 12-14, 66557 Illingen, Germany
		Telephone:	+49-6825-44071
		Fax number:	+49-6825-44073
		E-mail address:	Vetyl@t-online.de
1.2	**Manufacturer of Active Substance** (if different)	**Name:**	DuPont Chemoswed AB
		Address:	P.O. Box 839, Agneslungsvagen 27, S–201 80 Malmö, Sweden
		Telephone:	+46-40-383310
		Fax number:	+46-40-188971
		E-mail address:	claes.franzen-1@swe.dupont.com
1.3	**Manufacturer of Product(s)** (if different)	As given in Section A1.1.4 above.	

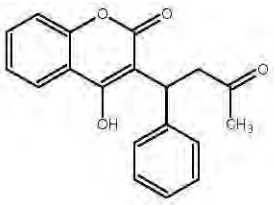
Section A2 Identity of Warfarin

Annex Point IIA2

Official use only

The active substance Warfarin is marketed in rodenticide products either as the "free acid" or as its Sodium salt. Since the active principle both under physiologically and environmentally relevant conditions is always the Warfarin molecule, the "free acid" and the Sodium salt should be considered biologically equivalent.

In this dossier, where relevant (i.e., for phys.-chem. properties), data are presented both for the "free acid" as well as the Sodium salt. However, when reviewing ecotoxicological and toxicological data, results of both the "free acid" and the Sodium salt are reviewed together. This is usually necessary since apart from an extensive experimental data base on Warfarin itself, a large proportion of studies was done with the Sodium salt for practical reasons, such as enhanced water solubility for aquatic toxicity testing and the use of the Sodium salt as the pharmaceutical product used in human clinical trials.

2.1	**Common name (IIA2.1)**	Name: Warfarin Synonyms: Athrombine-K, Brumalin, Coumadin, Coumafene (France), Dethmor, Kumatox, Rodafarin, Solfarin, Zoocoumarin (Russia), W.A.R.F. 42
2.2	**Chemical name (IIA2.2)**	IUPAC: (RS)-4-hydroxy-3-(3-oxo-1-phenylbutyl)coumarin 3-(α-acetonyl-benzyl)-4-hydroxycoumarin CA: 4-hydroxy-3-(3-oxo-1-phenylbutyl)-2H-1-benzopyran-2-one
2.3	**Manufacturer's development code number(s) (IIA2.3)**	No code numbers available.
2.4	**CAS No and EC numbers (IIA2.4)**	CAS: 81-81-2 [unspecified stereochemistry] 5543-58-8 [(R)-(+)-isomer] RTECS: GN 4550000 EINECS: 201-377-6 UN: 2476 CIPAC: 70
2.5	**Molecular and structural formula, molecular mass (IIA2.5)**	
2.5.1	Molecular formula	$C_{19}H_{16}O_4$
2.5.2	Structural formula	
2.5.3	Molecular mass	308.25 g/mol

Section A2 Identity of Warfarin

Annex Point IIA2

			Official use only
2.6	**Method of manufacture of the active substance (IIA2.6)**	Confidential information, please refer to Appendix I, Confidential data.	
2.7	**Specification of the purity of the active substance, as appropriate (IIA2.7)**	For detailed information on the purity specification of the active substance please refer to the 5 batch analyses for Warfarin and Sodium Warfarin in Appendix I, Confidential data. For verification of the racemic nature of the molecule Warfarin, please refer to the following report: **A2.7/01:** [redacted] 2001) Investigation of the optical activity of Warfarin by circular dichroism spectroscopy. [redacted] 22 October, 2001 (unpublished) For details on the nature of Coumadin (the commercial name of the pharmaceutical grade of Sodium Warfarin), please refer to the following document: **A2.7/02:** [redacted] (2001) Documentation on Coumadin. [redacted] January 2001, 19 pp.	
2.8	**Identity of impurities and additives, as appropriate (IIA2.8)**	Confidential information, please refer to Appendix I to Document III-A, Confidential data.	
2.9	**The origin of the natural active substance or the precursor(s) of the active substance (IIA2.9)**	Not applicable, since the active substance is synthesised from industrial chemicals.	

Section A2.10
Annex Point IIA2.10

Exposure data in conformity with Annex VIIA to Council Directive 92/32/EEC (OJ No L, 05.06.1992, p. 1) amending Council Directive 67/548/EEC

Subsection			Official use only
2.10.1	Human exposure towards active substance		
2.10.1.1	*Production*	See separate standard format (A2.10.1)	
2.10.1.2	*Intended use(s)*	Human exposure during use is considered to be product-related. Thus, exposure estimates are provided in Document III-B, Section 6.6 (Information related to the exposure of the biocidal product).	
2.10.2	Environmental exposure towards active substance		
2.10.2.1	*Production*		
	(i) Releases into water	No data available	
	(ii) Releases into air	No data available	
	(iii) Waste disposal	No data available	
2.10.2.2	*Intended use(s)*	PT 14 (Rodenticides), pest control in open areas, in and around buildings, sewage systems, and landfill sites.	
	Affected compartments:	Mackay model: Reference **A2.10.2/01**: [redacted](2004) Estimation of distribution in the environment of Warfarin. [redacted] February 12, 2004 (unpublished).	
	Water	99.5 %	
	Sediment	9.82×10^{-3} %	
	Air	1.08×10^{-8} %	
	Soil	0.442 %	
	Predicted concentration in the affected compartments	Predicted environmental concentrations are provided at Document II-B level.	
	Water	See Document II-B	
	Sediment	See Document II-B	
	Air	See Document II-B	
	Soil	See Document II-B	

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
Date **Materials and Methods** **Results and discussion** **Conclusion** **Reliability** **Acceptability** **Remarks**	EVALUATION BY RAPPORTEUR MEMBER STATE
Date **Materials and Methods** **Results and discussion** **Conclusion** **Reliability** **Acceptability** **Remarks**	COMMENTS FROM ...

Company Name	Name of A.S.	Month/Year
Warfarin Task Force	Warfarin, Sodium Warfarin	02/2004

Section A2.10 **Annex Point IIA2.10**	**Exposure data in conformity with Annex VIIA to Council Directive 92/32/EEC (OJ No L, 05.06.1992, p. 1) amending Council Directive 67/548/EEC**

Official use only

Subsection

2.10.1 Human exposure towards active substance

The following form requests information about occupational exposure towards the active substances based on Annex VIIA to Council Directive 92/32/EEC (OJ No L, 05.06.1992, p. 1) amending Council Directive 67/548/EEC.

Further information of the Technical Guidance Document in Support of Commission Directive 93/67/EEC on Risk Assessment for New Notified Substances, Commission Regulation (EC) No 1488/94 on Risk Assessment for Existing Substances (short title: TGD for Risk Assessment for New and Existing Substances) was taken into account.

The detailed structure supports the company to avoid further requests for the required data.

2.10.1.1 Production

2.10.1.1.1 Likely tonnage to be placed on the market per year [IIA V.5.8]

[***Note:*** *This field is taken from section IIA V.5.8 and must be filled in only in this chapter. This option will be available only in the electronic form*]

Produced	☒
Imported	☐
Quantity lower	0.2
Quantity upper	0.2
Unit (Quantity)	tpy
Year	2004
Remarks / further specifications	Annual biocidal use based on data from the applicants indicates that the annual volume of Warfarin contained in rodenticide products amounts to a total of 200 kg per year.

2.10.1.1.2 Description of process

Temperature of process	no data available
Pressure of process	no data available
Use pattern	no data available
Type of process	no data available
Batch size	no data available
Throughput	no data available

Company Name	Name of A.S.	Month/Year

Further description of process	no data available
Remarks / further specifications	no data available
2.10.1.1.3 Workplace description	no data available
Pattern of control	*In the following section describe the actual used pattern of control.*
Engineering controls	no data available
Administrative procedures	no data available
Personal protective equipment	no data available
Remarks / further specifications	

Company Name	Name of A.S.	Month/Year

2.10.1.1.4 Exposure

2.10.1.1.4.1 Task	no data available
	Note: *If more than one task is indicated fill in the fields of inhalation and dermal exposure for each task. Please use the field below "Further Task?" (end of 2.10.1.1.4.1.2) which support your fill in procedure.*
2.10.1.1.4.1.1 Inhalationexposure	no data available
Description of method	no data available
Frequency of task(s)	no data available
Duration of task(s)	no data available
Form during handling	no data available
Year(s) of measurement	no data available
Number of measurements	no data available
Type of measurements	no data available
Exposure concentration	no data available
Typical case	no data available
Reasonable worst case	no data available
Remarks	no data available
2.10.1.1.4.1.2 Dermal exposure	no data available
Description of method	no data available
Frequency of task	no data available
Duration of task	no data available
Form during handling	no data available
Exposed parts of the body	no data available
	(Reference: Risk assessment for occupational dermal exposure to chemicals, RISKOFDERM. Contract QLK4-CT-1999-01107, Part 1)
Year(s) of measurement	no data available
Number of measurements	no data available
Type of measurements	no data available
	Note: *personal sampling is appropriate*
Exposure concentration	no data available
Typical case	no data available
Reasonable worst case	no data available
Remarks	no data available

Company Name	Name of A.S.	Month/Year

Further Task? ☐

Company Name	Name of A.S.	Month/Year

2.10.1.2 Intended uses

2.10.1.2.1 Use of active substance for the formulation of biocidal product

2.10.1.2.1.1 Likely tonnage to be placed on the market per year [IIA V.5.8]	see 2.10.1.1 above [*Note: this information is taken from section IIA V.5.8 and must be filled in only in the actual chapter. This option will be available only in the electronic form*]
Produced	☒
Imported	☐
Quantity lower	see 2.10.1.1 above
Quantity upper	see 2.10.1.1 above
Unit (Quantity)	see 2.10.1.1 above
Year	see 2.10.1.1 above
Remarks / further specifications	see 2.10.1.1 above

2.10.1.2.1.2 Description of process

Temperature of process	ambient
Pressure of process	ambient
Use pattern	inclusion into matrix
Type of process	batchwise
Batch size	no data available
Throughput	no data available
Further description of process	Warfarin is made into a pre-mix with food-grade materials, and then blended into the remaining constituents (dye, food base and binders) in a mixer (dust-proof, closed system). Only one person carries out this operation. Any spill during filling operations is directyl re-introduced into the mixer, and any dust formed is effectively removed by LEV.
Package details	The resulting finished product is packed either in 250 gram bags, or in 100 gram tins.
Site inventory	no data available

Company Name	Name of A.S.	Month/Year

Storage information	ambient
Concentration of marketed formulation	max. 0.08% (W/W) Warfarin
Remarks / further specifications	none

2.10.1.2.1.3 Workplace description

This desciption of the formulation process of cereal-based rodenticide baits pertains both to the formulation of Tox Vetyl-Fertigköder, as well as Tox-Vetyl Festköder.

The original references (description of productio process) for both products are considered to represent confidential information, and are therefore contained in the fileof confidential information.

References: A2.10.1/01 and A2.10.1/02 (in German language).

The tasks include:

- weighing of active ingredient (Warfarin) on a scale in a booth connected to an air extraction system, to generate a pre-mix

- adding the pre-mix to a manufacturing blender protected by air extraction (dust-proof)

- weighing 0.025% & 0.05% warfarin baits onto a scale in a protected booth for subsequent packaging.

- moving bagged/boxed bait from the scale to an area nearby for bag-closing and box-taping activity.

Pattern of control	*In the following section describe the actual used pattern of control.*
Engineering controls	LEV
Administrative procedures	Yes; not further specified
Personal protective equipment	The operator wears full PPE as follows: Tyvek overall, protective gloves, respirator, goggles
Remarks / further specifications	none

Company Name	Name of A.S.	Month/Year

2.10.1.2.1.4 Exposure

2.10.1.2.1.4.1 Task	Tasks are described in the workplace desccription (2.10.1.2.1.3 above). ***Note:*** *If more than one task is indicated fill in the fields of inhalation and dermal exposure for each task. Please use the field below "Further Task?" (end of 2.10.1.2.1.4.2) which support your fill in procedure.*
2.10.1.2.1.4.1.1 Inhalation exposure	no data available
Description of method	no data available
Frequency of task(s)	no data available
Duration of task(s)	no data available
Form during handling	solid
Year(s) of measurement	no data available
Number of measurements	no data available
Type of measurements	no data available
Exposure concentration	no data available
Typical case	no data available
Reasonable worst case	no data available
Remarks	Personal inhalation exposure measurements are not available for this site, but for another one formulating a similar product, but with lower Warfarin content (0.05%): A2.10.1/03: [redacted](2004) Report on warfarin-exposure assessments carried out between 1988 and 1993 [redacted] Report dated February 2, 2004 (unpublished). A2.10.1/04: [redacted](2004) Inhalation exposure toWarfarin during bait formulation [redacted] February 11, 2004 (unpublished).
2.10.1.2.1.4.1.2 Dermal exposure	no data available
Description of method	no data available
Frequency of task(s)	no data available
Duration of task(s)	no data available
Form during handling	no data available

Company Name	Name of A.S.	Month/Year

Exposed parts of the body	A calculation was not considered necessary, in view of the full PPE that is worn, so that dermal exposure is considered negligible, and no specific part of the body is exposed. *(Reference: Risk assessment for occupational dermal exposure to chemicals, RISKOFDERM. Contract QLK4-CT-1999-01107, Part 1)*
Year(s) of measurement	no data available
Number of measurements	no data available
Type of measurements	no data available ***Note:** personal sampling is appropriate*
Exposure concentration	no data available
Typical case	no data available
Reasonable worst case	no data available
Remarks	no data available
Further Task?	☒

Section A3 Physical and Chemical Properties of Warfarin

Subsection (Annex Point)	Method	Purity/ Specification	Results	Remarks/ Justification	GLP (Y/N)	Reliability	Reference	Official use only
3.1 Melting point, boiling point, relative density (IIA3.1)								
3.1.1 Melting point	OECD 102 and EC A.1 (differential scanning calorimetry)	Batch no. 52200895, purity: 100.39 %	Melting temperature: 165°C (438 K) Comment: the melting process started at a lower temperature of 159°C (432 K)		Yes	1	**A3.1.1/01:** [redacted] (1998) Determination of the melting temperature of Warfarin technical. [redacted] September 1998 (unpublished)	
			Melting point 161-162°C		Not applicable	2	**A3.1.1/02:** Agrochemicals Handbook, 11th Ed.	
3.1.2 Boiling point	Justification for non-submission: The boiling point of Warfarin was calculated to 494 °C (reference 3.1.2/01), which will be above the observation limit of 360 °C. Furthermore the decomposition or a reaction of Warfarin was observed at and above 290 °C in a DSC study for the determination of the melting point (reference 3.1.1/01). This leads to the conclusion that the conduct of a study for the determination of the boiling point is not appropriate.							

Section A3 Physical and Chemical Properties of Warfarin

Subsection (Annex Point)		Method	Purity/ Specification	Results	Remarks/ Justification	GLP (Y/N)	Reliability	Reference	Official use only
		Guideline: not stated Differential scanning calorimetry (DSC)	Batch no. 52200895, purity: 100.39 %	No boiling point was observed in a DSC run under atmospheric pressure up to a temperature of 312 °C (585 °K) Instead, an exothermic effect above 290 °C was assumed to be caused by decomposition or reaction.	The result is adopted from the study on the melting point (A3.1.1/01). Since DSC is a valid method for determination of a boiling point, compliance to OECD 103 and EC method A.2 is given.	Yes	1	Cross-reference: **A3.1.1/01**	
		QSAR model calculation using "MpBpWin Program"	Not applicable	Estimated boiling point: 494.37 °C (1011.96 °K)		Not applicable	1	**A3.1.2/01:** [redacted](2004) Model calculation of boiling point for Warfarin. [redacted] (unpublished)	
3.1.3	Bulk density/ relative density	OECD 109 and EC A.3	Batch no. 52200895, purity: 100.39 %	Density at 20°C = 1.35 g/cm^3; D_4^{20} =1.35		Yes	1	**A3.1.3/01:** [redacted](1998) Determination of the density of Warfarin technical. [redacted] September 1998 (unpublished)	

Section A3 Physical and Chemical Properties of Warfarin

Subsection (Annex Point)	Method	Purity/ Specification	Results	Remarks/ Justification	GLP (Y/N)	Reliability	Reference	Official use only
3.2 Vapour pressure (IIA3.2)	OECD 104 and EC A.4		The vapour pressure was extrapolated from a measurement with Warfarin and was determined to be p (20°C) ≤ 3×10^{-5} hPa.		Yes	1	**A3.2/01:** [redacted] (2001) Warfarin – Determination of the vapour pressure. [redacted] August 31, 2001 (unpublished)	
	QSAR model calculation		The vapour pressure was calculated to a value of 4.6×10^{-10} Pa at 20 °C.		Not applicable	1	**A3.2/02:** [redacted] (1999) Model calculation of the vapour pressure of Warfarin. [redacted] April 20, 1999 (unpublished)	
			9×10^{-2} mbar at 21.5 °C.		Not applicable	2	Cross-reference: **A3.1.1/02** Agrochemicals Handbook, 11th Ed.	
3.2.1 Henry's Law Constant (IIA3.2)	QSAR model calculation		H = 2×10^{-4} Pa × m^3/mol at 20 °C		Not applicable	1	**A3.2.1/01:** [redacted] (1998) Model calculation of Henry's Constant. [redacted] June 11, 1998 (unpublished)	
3.3 Appearance (IIA3.3)								
3.3.1 Physical state			solid, powder					

Section A3 Physical and Chemical Properties of Warfarin

Subsection (Annex Point)	Method	Purity/ Specification	Results	Remarks/ Justification	GLP (Y/N)	Reliability	Reference	Official use only
3.3.2 Colour			White					
3.3.3 Odour			odourless, tasteless					
3.4 Absorption spectra (IIA3.4)		Batch no: 10124, purity: 98 %			Yes	1	**A3.4/01:** [redacted](2001) UV/VIS Absorption Spectrum, Infrared Absorption-Spectrum; H-NMR Spectrum and Mass Spectrum of Warfarin. [redacted] August 12, 2001 (unpublished)	
UV/VIS	OECD 101		Please refer to Figure A3-1 to Figure A3-2 and Table A3-1 to Table A3-2.					
IR			Please refer to Figure A3-3 and Table A3-3.					
NMR			Please refer to Figure A3-4 to Figure A3-5 and Table A3-4.					
MS			Please refer to Figure A3-6 and Figure A3-7.					

Section A3 Physical and Chemical Properties of Warfarin

Subsection (Annex Point)	Method	Purity/ Specification	Results	Remarks/ Justification	GLP (Y/N)	Reliability	Reference	Official use only
3.5 Solubility in water (IIA3.5)	OECD 105 and EC A.6	Batch no. 52200895, purity: 100.39%.	65.9 g/l [8°C, at pH=9.06] 264 mg/l [8°C, at pH=7.42] 2.9 mg/l [8°C, at pH=4.07] 66.13 g/l [20°C, at pH=9.14] 267 mg/l [20°C, at pH=7.12] 4.9 mg/l [20°C, at pH=4.07] 69.44 g/l [30°C, at pH=8.86] 285 mg/l [30°C, at pH=6.89] 7.3 mg/l [30°C, at pH=4.05] 17 mg/l (20°C)	Comment: the test solutions needed to be buffered with carbonate at neutral and alkaline pH to avoid drifting of the pH.	Yes	1	**A3.5/01:** [redacted](1998) Water solubility of Warfarin. [redacted] November 10, 1998 (unpublished) Cross-reference: **A3.1.1/02** Agrochemicals Handbook, 11th Ed.	
3.6 Dissociation constant (-)	OECD guideline 112	Batch no. 10124, purity: 98 %	pK_a = 5.19 (20°C)		Yes	1	**A3.6/01:** [redacted](2003) Ionisation Constant of Warfarin in Water. [redacted] February 24, 2003 (unpublished)	

Section A3 Physical and Chemical Properties of Warfarin

Subsection (Annex Point)	Method	Purity/ Specification	Results	Remarks/ Justification	GLP (Y/N)	Reliability	Reference	Official use only
3.7 Solubility in organic solvents, including the effect of temperature on solubility (IIIA3.1)	In adaptation of OECD 105 and EC A.6	Batch no. 52200895, purity: 100.39 %	Please refer to Table A3-5.		Yes	1	**A3.7/01:** [redacted] (1998) Solubility of Warfarin in organic solvents. [redacted] November 11, 1998 (unpublished)	
			Please refer to Table A3-5.		Not applicable	2	Cross-reference: **A3.1.1/02** Agrochemicals Handbook, 11th Ed.	
3.8 Stability in organic solvents used in b.p. and identity of relevant breakdown products (IIIA3.2)			Not relevant, only required if organic solvents are included in the biocidal product.	waiver				

Section A3 Physical and Chemical Properties of Warfarin

Subsection (Annex Point)	Method	Purity/ Specification	Results	Remarks/ Justification	GLP (Y/N)	Reliability	Reference	Official use only
3.9 Partition coefficient n-octanol/water (IIA3.6)	OECD 107 and EC A.8	Batch no.: 070899A351, purity: 99.44 %	log P_{ow} (calculated): 2.9 (pH 4) 0.7 (pH 7) 0.6 (pH 9).		Yes	1	**A3.9/01:** [redacted] (2001) Determination of the Partition Coefficient (n-Octanol/Water) of Warfarin technical by High Performance Liquid Chromatography (HPLC). [redacted] October 02, 2001 (unpublished)	
	OECD 117, EC A.8 Deviations: photometric analysis of Warfarin in an octanol phase which was previously equilibrated with aqueous solutions buffered at various pH values.	Warfarin, recryst. from aqueous acetone	log P_{ow} = 2.82 (21 °C) (non-dissociated). The measured absorbance of Warfarin in the octanol phase as a function of pH is presented in Figure A3-8 (the model fitted to equation (8A), k_1=0.258 and k_2=6.375*10^{-9}). The partitioning of anionic Warfarin into lipophilic media may be regarded as negligible, since in this investigation it was verified that no measurable partitioning of the ionised form into octanol phase was observed.		No	2	**A3.9/02:** Opong-Mensah K, Woller TW, Obaseki AO, Porter WR (1984) Chemical and statistical considerations in the determination of partition coefficients of weakly ionisable drugs and poisons. J. Pharm. Biomed. Anal. 2, 381-394 (published)	

Section A3 Physical and Chemical Properties of Warfarin

Subsection (Annex Point)	Method	Purity/ Specification	Results	Remarks/ Justification	GLP (Y/N)	Reliability	Reference	Official use only
			$\log P_{ow}$ = 3.28 (25 °C)		No	2	**A3.9/03:** van der Giesen WF, Janssen LHM (1982) Influence of ionization and ion-pair formation on lipophilicity of some 4-hydroxycoumarin derivatives in the octanol-water system. Int. J. Pharm. 12, 231-249 (published)	
3.10 Thermal stability, identity of relevant breakdown products (IIA3.7)			The purpose of this test is to obtain preliminary judgement of the stability of a substance with respect to heat and air in order to provide guidance in the performance of other tests. However, in a DSC analysis (see 3.1.1./01 above), Warfarin has been shown to be stable up to at least 290°C, indicating adequate thermal stability under conditions of practical handling and use.	waiver			Cross-reference: **A3.1.1/01**	

Section A3 Physical and Chemical Properties of Warfarin

Subsection (Annex Point)	Method	Purity/ Specification	Results	Remarks/ Justification	GLP (Y/N)	Reliability	Reference	Official use only
3.11 Flammability, including auto-flammability and identity of combustion products (IIA3.8)	EC method A.10	Batch no. 52200895, purity: 100.39 %	Under the conditions of the test, Warfarin could not be ignited. Thus, Warfarin techn. is not „flammable“ according to the criteria of the test method/directive.		Yes	1	**A3.11/01:** [redacted] (1998) Determination of the flammability of Warfarin technical. [redacted] September 1998 (unpublished)	
	EC method A.16	Batch no. 52200895, purity: 100.39 %	No endothermic or exothermic reaction was observed up to a test temperature of 400 °C. Thus, Warfarin techn. is not “self-ignitable” according to the criteria of the test method/directive.		Yes	1	**A3.11/02:** [redacted] (1998) Determination of the relative self-ignition temperature of Warfarin technical. [redacted] September 1998 (unpublished).	
3.12 Flash-point (IIA3.9)			Not applicable. The performance of this test is not required, since Warfarin is a solid (melting point > 40 °C).	waiver				

Section A3 Physical and Chemical Properties of Warfarin

Subsection (Annex Point)	Method	Purity/ Specification	Results	Remarks/ Justification	GLP (Y/N)	Reliability	Reference	Official use only
3.13 Surface tension (IIA3.10)	OECD 115 and EC method A.5	Batch no. 52200895, purity: 100.39 %	not surface active: the surface tension of an aqueous solution of Warfarin technical at a 90 % saturated concentration was 72.8 mN/m (20 °C). For comparative purposes, the surface tension of double-distilled water was determined at 73.9 mN/m.		Yes	1	**A3.13/01:** [redacted] (1998) Determination of the surface tension of an aqueous solution of Warfarin technical. [redacted] September 1998 (unpublished)	
3.14 Viscosity (-)			No data are submitted, since Warfarin is solid and not liquid under atmospheric pressure and room temperature	waiver				

Section A3 Physical and Chemical Properties of Warfarin

Subsection (Annex Point)	Method	Purity/ Specification	Results	Remarks/ Justification	GLP (Y/N)	Reliability	Reference	Official use only
3.15 Explosive properties (IIA3.11)	EC A.14 Deviations: QSAR calculation using CHETAH (rev. 7.2), Chemical Thermodynamic and Energy Release Evaluation, ASTM Subcommittee E27.07 on Estimation Methods, ASTM DS51C.		The molecule Warfarin does not contain any structural groups known to be correlated with a particular explosion hazard. In addition, thermodynamic calculations (CHETAH) have shown that the heat of decomposition and the comparison to the heat of combustion do not necessitate explosivity to be expected. Finally, the oxygen balance is sufficiently negative not to expect any inherent dangerous properties in this context. In conclusion, structural and thermodynamic assessments lead to the conclusion that Warfarin must not be considered to have explosive properties.		Not applicable	1	**A3.15/01:** [redacted] (2004) Explosivity, Warfarin technical. [redacted] January 13, 2004 (unpublished)	

Section A3 Physical and Chemical Properties of Warfarin

Subsection (Annex Point)	Method	Purity/ Specification	Results	Remarks/ Justification	GLP (Y/N)	Reliability	Reference	Official use only
3.16 Oxidizing properties (IIA3.12)	EC A.17 Deviations: QSAR calculation using CHETAH (rev. 7.2), Chemical Thermodynamic and Energy Release Evaluation, ASTM Subcommittee E27.07 on Estimation Methods, ASTM DS51C.		According to the chemical structure of Warfarin, it can be ruled out that the active ingredient might react exothermally with combustible material. In addition, thermodynamic calculations (CHETAH) have shown that the composition of this molecule does not even allow combustion on its own, let alone an exothermic reaction with other combustible material. Finally, the oxygen balance is sufficiently negative not to expect any inherent dangerous properties in this context. In conclusion, structural and thermodynamic assessments lead to the conclusion that Warfarin must not be considered to have oxidising properties.		Not applicable	1	**A3.16/01:** [redacted] (2004) Oxidising Properties of Warfarin technical. [redacted] February 14, 2004 (unpublished)	
3.17 Reactivity towards container material (IIA3.13)	expert statement		Warfarin is neither an acidic, nor an alkaline substance. In addition, it does not have any oxidising properties. Based on experience gained in more than 5 decades of normal handling and use, there have been no reports of any reactivity towards container materials.					

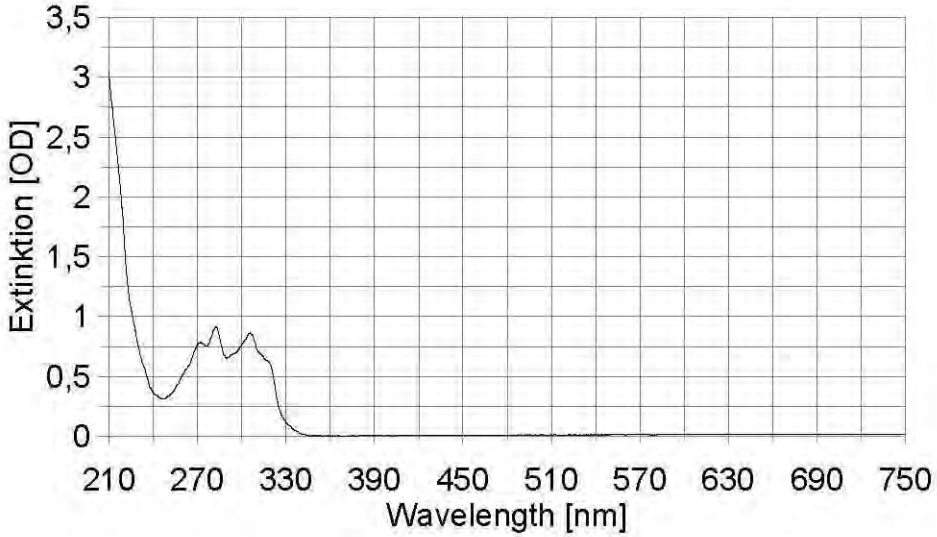


Figure A3-1: UV/VIS spectrum of 24.9 mg/L Warfarin at neutral pH.

Table A3- 1: Peak maxima/ minima and molar absorption extinction coefficient (ε) at neutral pH values at 24.9 mg/l.

	Min	Max	Min	Max	Min	Max	Min
λ [nm]	246.5	272	275.5	282.5	290	306	368.5
E [OD]	0.313	0.785	0.757	0.916	0.657	0.868	0.001
ε [l / mol / cm]	3875	9719	9372	11341	8134	10747	12

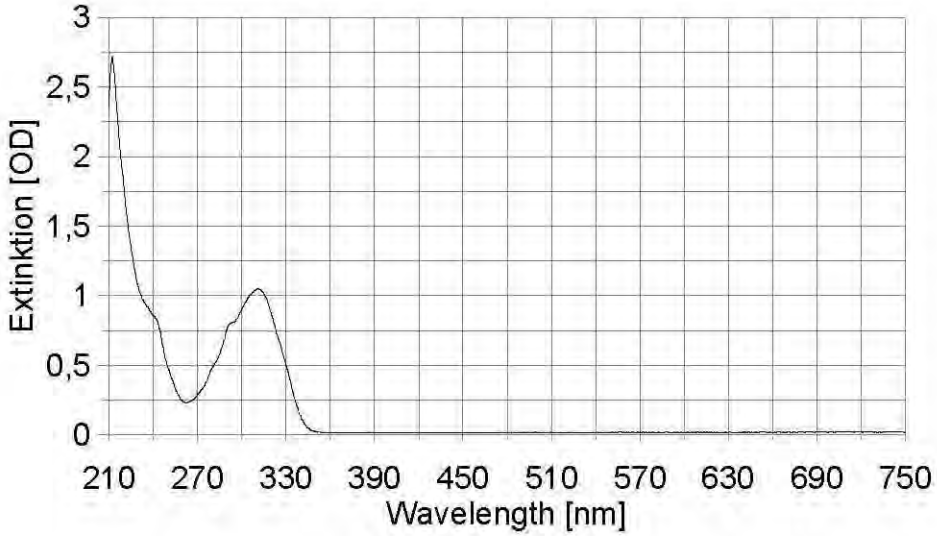


Figure A3-2:UV/VIS spectrum of 24.9 mg/L Warfarin at alkaline pH.

Table A3-2: Peak maxima / minima and molar absorption extinction coefficient (ε) at alkaline pH values at 24.9 mg/l.

	Max	Min	Max	Min	Max	Min
λ [nm]	212.5	262.5	293.5	294	311	345
E [OD]	2.719	0.233	0.809	0.808	1.052	0.052
ε [l / mol / cm]	33663	2885	10016	10004	13025	644

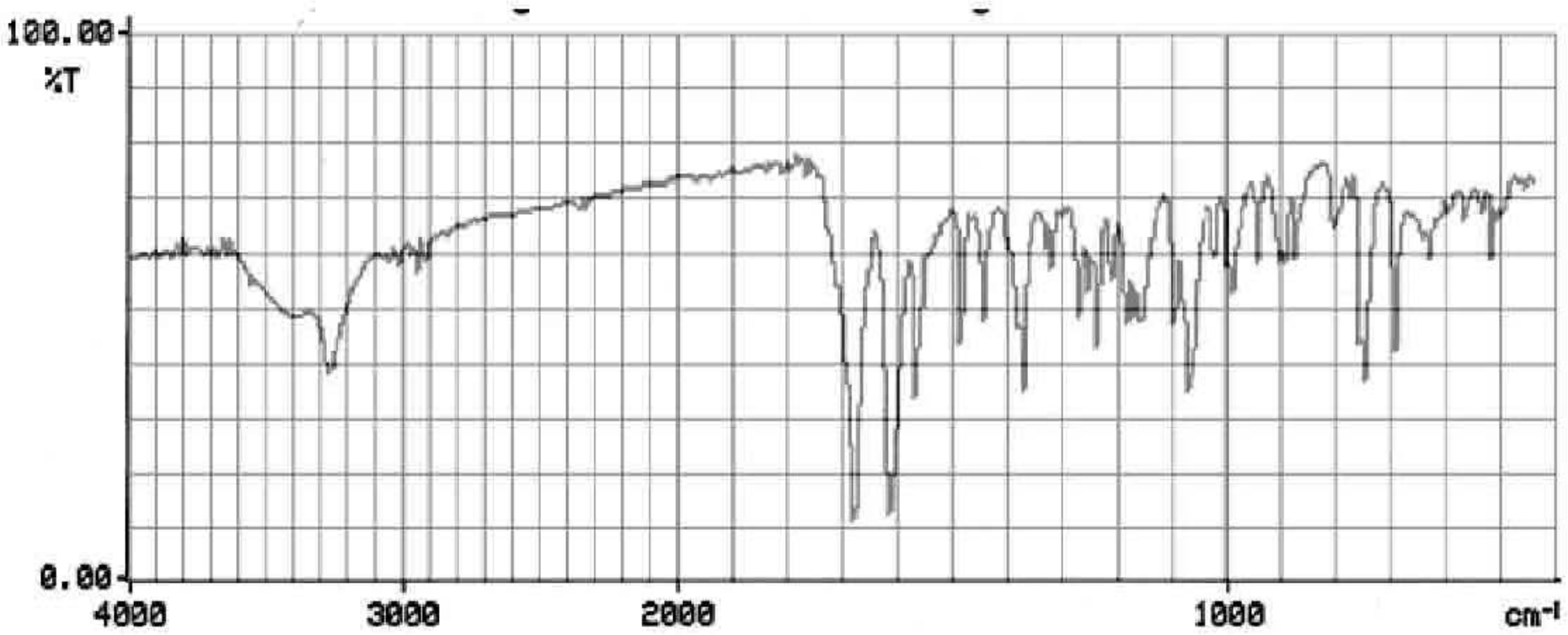


Figure A3-3:IR-Spectrum of Warfarin, range 4000-450 cm^{-1}.

Table A3- 3: Characteristic resonance of the IR-spectrum.

Wave number [cm^{-1}]	Group bands	Assignment
3600 - 3300	Intermolecular hydrogen bonds	OH stretching of intermolecular H bonds from Oxygen to aromatic protons
3000 -2800	Aliphatic	C-H stretching
1681	Aliphatic	C=O stretching
1617, 1493, 1452	Aromatic	All ring bonds stretching
1077 and 1030	Ring	C-O-C stretching
1600 to 800	overlay of carboxylic ester	C-H stretching, C-C stretching, C-O stretching
755 and 764	Aromatic	aromatic C-H bend of the ortho-substituted benzene and the mono-substituted benzene
701	Aromatic	ring C-C bend

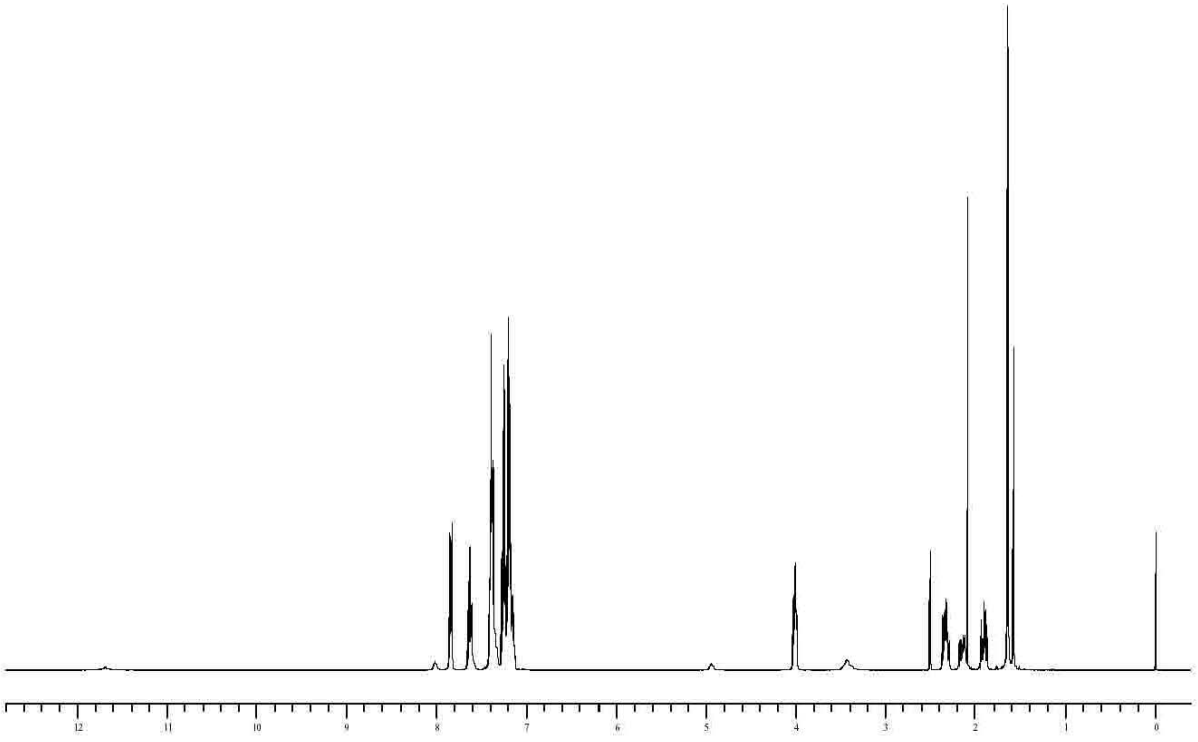


Figure A3- 4: H-NMR-spectrum of Warfarin (500 MHz; TMS at 0.000ppm).

Table A3-4: Protons and assigned shift in H-NMR.

Atom-Id.	^{1}H-chem. shift related to TMS [ppm]	Coupling constant J_{HH} [Hz]
1, 1'	7.373 doublet	7.03/ 8.03
2, 2'	7.638 triplet	7.36/ 8.03
3, 3'	7.411 triplet	3.01/ 4.35
4, 4'	7.845 doublet	7.03
5	–	None
6	–	None
7	–	None
8	–	None
9	–	None
10	–	None
11, 11'	Possible different singulets after uptake of proton 11.69, 8.023, 4.94 and 3.411 (possible water in DMSO)	
12, 12'	1.908 triplet, doublet (after loss of CH acidic proton)	12.38/ 12.72
13, 13'	different singulets, 11.69, 8.023, 4.94 and 3.411 (possible water in DMSO)	
14, 14'	4.000 triplet mixed with doublet after loss of CH acidic proton	6.69/ 7.03/ 7.36
15	–	None
16, 16'	different singulets, 1.584 CH_3 and 1.653 CH_2 after loss of CH acidic proton	None
17	–	None
18, 18'	7.256 doublet	7.36/ 5.69
19, 19'	7.199 triplet	7.36/ 7.70
20, 20'	7.147 triplet	7.36
21, 21'	7.199 triplet	7.36/ 7.70
22, 22'	7.256 doublet	7.36/ 5.69
23, 23'	Possible different singulets after uptake of proton 11.69, 8.023, 4.94, 3.411 (possible water in DMSO) and 2.008	

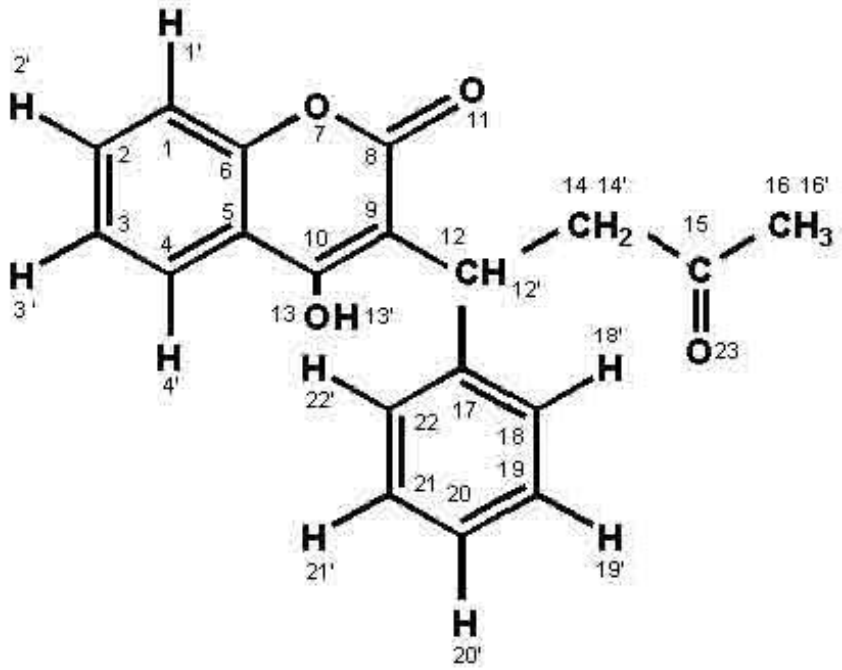


Figure A3-5: Molecular structure and proton position.

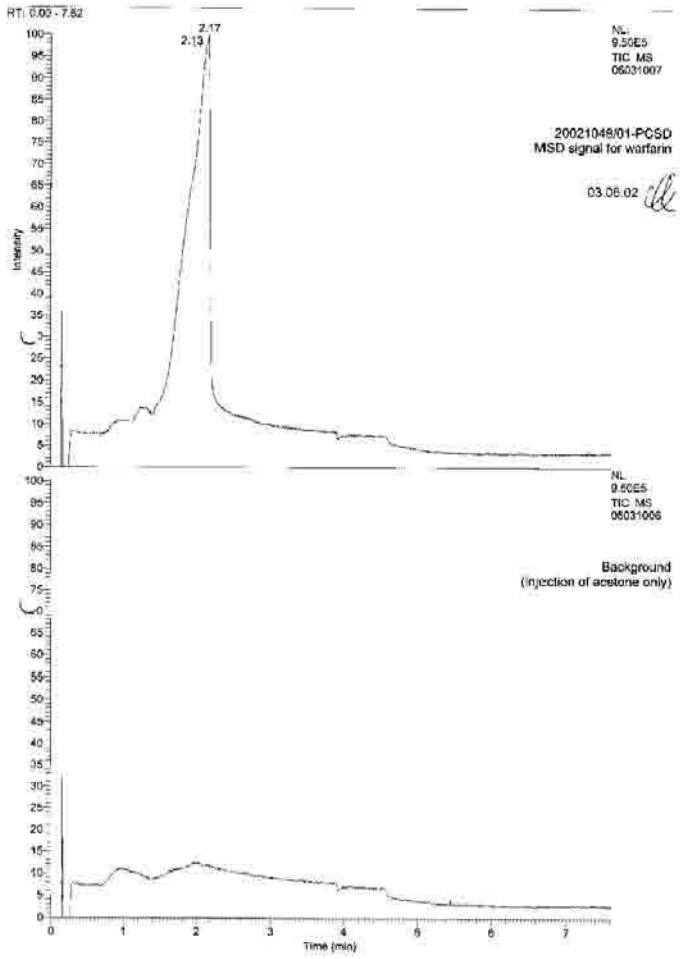


Figure A3-6: GC/MS-chromatogram of Warfarin with a retention time of 11.75 min.

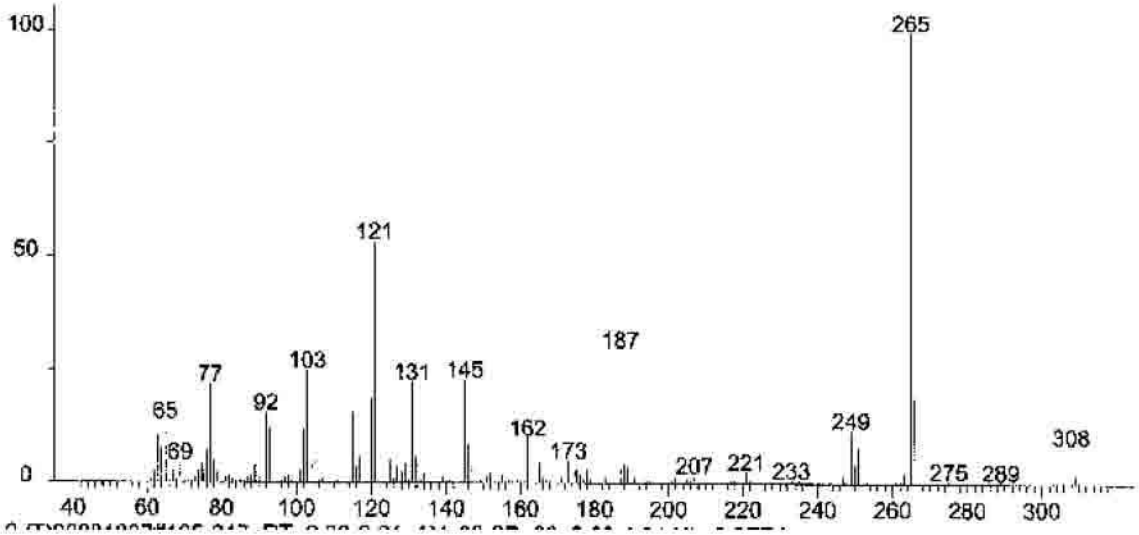


Figure A3-7: MS-spectrum of Warfarin, 11.75 min, 70eV.

Table A3- 5: Solubility in Organic solvents.

Solvent	Solubility at 20°C
Data from reference A3.7/01 (GLP)	
n-Heptane	6.41 ± 0.11 mg/l
Xylol	780 ± 20 mg/l
1,2-Dichloroethane	21.19 ± 1.07 g/l
Methanol	22.15 ± 0.23 g/l
Acetone	54.59 ± 0.94 g/l
Ethyl acetate	16.93 ± 0.13 g/l
Data from reference A3.7/02	
Acetone	65 g/l
Benzol	only very slightly
Chloroform	56 g/l
Cyclohexane	only very slightly
Diethylether	only very slightly
Dioxane	100 g/l
Methanol	moderately soluble

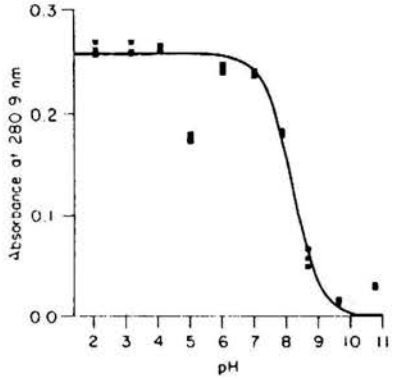


Figure A3- 8: Absorbance of Warfarin in the octanol phase as a function of pH.

Section A4.1
Annex Point IIA4.1

Analytical Methods for Detection and Identification
Technical product

Official use only

1 REFERENCE

1.1	**Reference**	**A4.1/01:** [redacted](2003) Validation of a confirmatory method for analysis of Warfarin in Warfarin Technical grade material. [redacted] February 19, 2003 (unpublished)
1.2	**Data protection**	Yes
1.2.1	Data owner	Warfarin Task Force
1.2.2	Companies with letter of access	Spiess Urania Chemicals GmbH, Hamburg, Germany; Killgerm Chemicals Ltd., Osset, UK; Hentschke & Sawatzki KG, Neumünster, Germany. Vetyl Chemie GmbH, Illingen, Germany
1.2.3	Criteria for data protection	Data on existing a.s submitted for the first time for entry into Annex I of Directive 98/8/EEC.

2 GUIDELINES AND QUALITY ASSURANCE

2.1	**Guideline study**	Yes, SANCO/3030/99 rev.4
2.2	**GLP**	Yes
2.3	**Deviations**	None

3 MATERIALS AND METHODS

3.1	**Preliminary treatment**	None
3.2	**Detection**	
3.2.1	Separation method	HPLC/MS-MS (reversed-phase column; mobile phase: acetonitrile/water/formic acid (50:50:0.2))
3.2.2	Detector	LCQ Duo Ion Trap System, electro spray interface (negative polarity), monitored ions: m/z = 307 –> 161
3.2.3	Test substance	Warfarin, batch no.: 010598A351, purity: 100%
3.2.4	Reference substance	Warfarin, batch no.: 10124, purity: 98.0%
3.3	**Linearity**	
3.3.1	Calibration range	The detector response was linear for standard solutions in the concentration range of 25 to 200 ng/mL.
3.3.2	Number of measurements	5 different concentrations

Section A4.1 **Analytical Methods for Detection and Identification**
Annex Point IIA4.1 **Technical product**

3.3.3	Linearity	y = 400279 + 14259.2 x; r^2 = 0.9916 y = the response in the chromatogram and x = the concentration of the substance [ng/mL].
3.4	**Specificity: interfering substances**	No interfering substances were observed. The present study enables the specific determination of Warfarin. Due to the combination of two MS steps, the negatively charged molecular ion at m/z = 307 and fragment ion at m/z = 161 were detected after chromatographic separation (retention time = 5.1 min). A minimum of 3 ions as it is required by the SANCO 3030/99/rev.4 for identification and quantification is not applicable to HPLC/MS-MS analysis.
3.5	**Recovery rates at different levels**	Accuracy was determined by analysis of five subsets of technical Warfarin of one batch with known amounts of pure a.s. on the total amount of Warfarin. The recovery was 98.1 %.
3.5.1	Relative standard deviation	RSD = 1.3 %.
3.6	**Limit of determination**	Not stated, only required for impurities.
3.7	**Precision**	
3.7.1	Repeatability	The precision of the method was not determined according to SANCO/3030/99 rev.4.
3.7.2	Independent laboratory validation	The repeatability of the method was determined by analysis of five subsets of technical Warfarin in accordance with SANCO 825-00/rev.6. RSD = 1.3 %, n = 5

4 APPLICANT'S SUMMARY AND CONCLUSION

4.1	**Materials and methods**	LC-MS/MS method for the determination of Warfarin in technical Warfarin.
4.2	**Conclusion**	The results of linearity, accuracy and specificity demonstrate that the analytical method is adequate and suitable for the determination of Warfarin in technical Warfarin, according to SANCO/3030/99 rev. 4.
4.2.1	Reliability	2
4.2.2	Deficiencies	The precision of the method was not strictly determined according to SANCO/3030/99 rev.4. The acceptability of the relative standard deviation was not assessed by application of the Horwitz equation as recommended by SANCO/3030/99 rev.4.

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
Date **Materials and Methods** **Results and discussion** **Conclusion** **Reliability** **Acceptability** **Remarks**	EVALUATION BY RAPPORTEUR MEMBER STATE
Date **Materials and Methods** **Results and discussion** **Conclusion** **Reliability** **Acceptability** **Remarks**	COMMENTS FROM ...

Section A4.1 Analytical Methods for Detection and Identification
Annex Point IIA4.1 **Technical product**

Supportive data

The following data were already submitted in the context of an application for inclusion of the active substance Warfarin in Annex I of Directive 91/414/EEC. Since these data are not considered to represent key studies, they are cited below as supportive data only:

Reference A4.1/02: [redacted] (1995) Specification and routine tests for active substances. [redacted] March 14, 1995 (unpublished)

This collection of methods represents the quality control test performed on the product as manufactured. It encompasses an HPLC assay for the content of Warfarin in the technical grade active ingredient, and a GC assay for the impurity Benzalacetone:

(I) HLPC Assay for Warfarin content in technical grade active ingredient

A sample (ca. 40 grams) of techn. grade Warfarin is dissolved in 40 ml of 0.1 M sodium hydroxide solution. To this, 25 ml of potassium dihydrogenphosphate, and the volume is adjusted to 100 ml with milli-Q water. To 5 ml of this solution, 7.5 ml of a 0.2 M potassium phosphate buffer solution (pH=7.4) are added, and the volume is adjusted to 25 ml with acetonitrile. Solutions of pure Warfarin prepared as above are used as external standards (n=3). Analysis is by isocratic (methanol/water/acetic acid 62:38:1) reversed phase HPLC on µ-Bondapak C18 with UV detection (282 nm).
The analysis is linear over a range from 0-150% of the estimated value. Precision (n=15) analysis yielded a mean value of 100.5%, with a repeatability of 0.91% and a reproducibility of 1.48%.

(II) GC assay for the impurity Benzalacetone

An accurately weighed amount (2 grams) of techn. grade Warfarin is dissolved in 1M sodium hydroxide solution (10 ml). To this, 1 ml of internal standard solution (250mg/l acenaphthene) and 9 ml of methylene chloride are added. After vigorous shaking and subsequent phase separation, the methylene chloride phase is filtered through a pad of cotton gauze. Analysis is by GC/FID.
The method is linear for a range of 50-150 ppm of benzalacetone in the sample tested, with a precision of 1.55% (RSD) (n=13). The detection limit for benzalacetone is given at 2 ppm.

Section A4.1
Annex Point IIA4.1

Analytical Methods for Detection and Identification
Technical product
Supportive data

The following data were already submitted in the context of an application for inclusion of the active substance Warfarin in Annex I of Directive 91/414/EEC. Since these data are not considered to represent key studies, they are cited below as supportive data only:

Reference A4.1/03: [redacted] (1999) Analysis of 5 batches of Warfarin technical products, and of 2 batches of Warfarin Sodium salt technical products. [redacted] June 14,1999 (unpublished)

This GLP report describes the analysis of the Warfarin content in technical grade "Warfarin" and "Warfarin Sodium", plus the analysis for the impurities listed in Appendix I point 4.1 (see file of confidential information, section 12):

Warfarin:
A sample (ca. 50 mg) of Warfarin or Sodium Warfarin is accurately weighed, and then dissolved in a defined volume of isooctane/methylene chloride/methanol/glacial acetic acid (70:24:3:3 v/v). Analysis is by isocratic (mobile phase: heptane/methylene chloride/methanol/glacial acetic acid 89:8:1:2 v/v) reversed phase HPLC (method: METWARF) with diode array detection at 306 nm (for Warfarin).

The validation characteristics are as follows:

Specificity:	checked by injection of extracts from "blank" formulations, no interfering peaks
Linearity:	detector response is linear between 15.6 and 156 µg/ml, corresponding to a range between 0.16 and 1.6 % Warfarin in tracking powder
Accuracy:	checked by triplicate spiking of a "blank" formulation at three different fortification levels (0.02%, 0.04% and 0.08%)
Recovery:	98.5% for all three fortification levels; RSD: 1.5% (n=9)
Repeatability:	checked by 6 separate determinations of Warfarin content in samples,

mean value: 0.042 %; RSD: 2.79 % (n=6)

Impurities:
A sample (ca. 250 mg) of Warfarin or Sodium Warfarin is accurately weighed, and then dissolved in a defined volume of acetonitrile/water (90:10 v/v). Analysis is by gradient (water/methanol, both with 0.025 85% H_3PO_4) reversed phase HPLC (method: METIMPWF) with diode array detection at 290 nm. The detection limit for both impurities is given at 0.1 g/kg

Section A4.1 Analytical Methods for Detection and Identification
Annex Point IIA4.1 **Technical product**

Supportive data

The following data were already submitted in the context of an application for inclusion of the active substance Warfarin in Annex I of Directive 91/414/EEC. Since these data are not considered to represent key studies, they are cited below as supportive data only:

Reference A4.1/04: Raw GR (Ed.) (1970) Analysis of technical and formulated pesticides: Warfarin, and Warfarin, Sodium salt, CIPAC Handbook, Vol. 1, 696 - 702 (published)

This method represents the currently available CIPAC test methods for photometric assays of Warfarin and Sodium Warfarin. In the case of Warfarin, the sample is dissolved in chloroform, the resulting solution is chromatographed on an aluminium oxide column, and the UV absorbance is read at 305.5 nm. Sodium Warfarin samples are dissolved in water, acidified with 2 N sulphuric acid, and then extracted with chloroform, but the procedure is otherwise as described above.
The specificity of this method is questionable according to current standards. There is no data on linearity, precision and repeatability.

Section A4.1 Analytical Methods for Detection and Identification
Annex Point IIA4.1 **Technical product**

Supportive data

The following data were already submitted in the context of an application for inclusion of the active substance Warfarin in Annex I of Directive 91/414/EEC. Since these data are not considered to represent key studies, they are cited below as supportive data only:

Reference A4.1/05: D'Hulst A, Verbeke N (1994) Quantitation in chiral capillary electrophoresis: Theoretical and practical considerations. Electrophoresis 15, 854-863 (published)

A description of this method is included here because (i) it represents an alternative to other methods involving HPLC on chiral columns matrices, and (ii) it is an example of verification that commercially available Warfarin is in fact a true racemate.

The analytical system consists of a semi-automated capillary electrophoresis apparatus (Waters) operate at constant voltage (30 kV) using normal polarity mode. The separation is performed in a 50 µm ID and 60 cm length fused silica capillary, in an electrolyte composed of 2.5% Glucidex and 10 mM Tris-phosphate pH=7. Detection of analytes is by UV detection (214/185 nm) at the cathodic end.
Whereas validation data of this method are given in the publication for other drugs, there is no data on linearity, precision and repeatability for the analysis of Warfarin.

The following references identified in a literature search to contain information on Warfarin were considered to lack in relevance upon evaluation, and were thus omitted from this summary:

Author (year)	Title	EBRC ref. no.
Krause RT (1983)	Determination of fluorescent pesticides and metabolites by reversed-phase high-performance liquid chromatography	14
Niederlaender HAG, Gooijer C, Velthorst NH (1994)	Chemiluminescence detection in liquid chromatography based on photo-oxygenation involving reactive oxygen intermediates	28
Wong YWJ, Davis PJ (1989):	Analysis of warfarin and its metabolites by reversed-phase ion-pair liquid chromatography with fluorescence detection	48
Spink DC, Aldous KM, Kaminsky LS (1989):	Analysis of oxidative warfarin metabolites by thermospray high-performance liquid chromatography/ mass spectrometry	44
Vanhaelen-Fastre R, Vanhaelen M (1976)	High-performance liquid and thin-layer chromatography of coumarin anticoagulants and their degradation products	318

Section A4.2 **Analytical Methods for Detection and Identification in (a) soil**
Annex Point IIA4.2

Official use only

1 REFERENCE

1.1	**Reference**	**A4.2/01:** [redacted] (2001) Residue analysis of Warfarin in soil – method validation. [redacted] August 15, 2001 (unpublished).
1.2	**Data protection**	Yes
1.2.1	Data owner	Neudorff GmbH, Germany
1.2.2	Companies with letter of access	Spiess Urania Chemicals GmbH, Hamburg, Germany; Killgerm Chemicals Ltd., Osset, UK; Hentschke & Sawatzki KG, Neumünster, Germany. Vetyl Chemie GmbH, Illingen, Germany
1.2.3	Criteria for data protection	Data on existing a.s submitted for the first time for entry into Annex I of Directive 98/8/EC.

2 GUIDELINES AND QUALITY ASSURANCE

2.1	**Guideline study**	Yes SANCO/825/00/rev. 6
2.2	**GLP**	Yes
2.3	**Deviations**	None

3 MATERIALS AND METHODS

3.1	**Preliminary treatment**	
3.1.1	Enrichment	Residues of Warfarin were extracted with acetonitrile/water/acetic acid (90:10:0.1) after addition of sodium chloride. After evaporation the residues were reconstituted in HPLC mobile phase.
3.1.2	Cleanup	No further clean up was performed, since LC-MS/MS was used for quantification.
3.2	**Detection**	
3.2.1	Separation method	LC–MS/MS (reversed-phase column; mobile phase: acetonitrile/water/acetic acid, gradient)
3.2.2	Detector	LCQ Duo Ion Trap System, electro spray interface (negative polarity), monitored ions: m/z = 308 –> 161.
3.2.3	Standard(s)	Warfarin, batch no. 60205, purity: 99.9% Soil sample: pH 6.8, dry matter: 93%; total organic carbon: 10.5 g/kg dry substance.
3.2.4	Interfering substance(s)	No interfering substances were observed.

Section A4.2 **Analytical Methods for Detection and Identification in (a) soil**
Annex Point IIA4.2

3.3	**Linearity**	
3.3.1	Calibration range	The detector response for the analytical standards was second order (quadratic) in the range of 10 ng/ml to 250 ng/ml.
3.3.2	Number of measurements	The calibration curve was plotted based on seven different concentrations.
3.3.3	Linearity	The equation of a typical standard calibration function for Warfarin was determined as $y = 386671 + 259595x - 315.042x^2$; $r^2 = 0.9956$ where y is the response in the chromatogram and x the concentration of the substance [ng/ml].
3.4	**Specificity: interfering substances**	The method enables the specific determination of Warfarin in soil. No interfering substances were observed. The method is highly specific, since MS/MS was used for detection.
3.5	**Recovery rates at different levels and relative standard deviations**	Please refer to Table A.4.2-1.
3.6	**Limit of quantification**	The limit of quantification was 0.02 mg/kg.
3.7	**Precision**	
3.7.1	Repeatability	The recovery rates were in a range of 70 – 110 % and the relative standard deviations were below 20% at each fortification level.
3.7.2	Independent laboratory validation	Not required.

4 APPLICANT'S SUMMARY AND CONCLUSION

4.1	**Materials and methods**	Residues of Warfarin were extracted with acetonitrile/water/acetic acid (90:10:0.1). Determination was performed by LC-MS/MS.
4.2	**Conclusion**	The average recoveries were in the range of 70 – 110% with relative standard deviations below 20%. No interfering blanks were observed. Therefore, this method fulfils the requirements of SANCO/825/00 rev. 6 as an enforcement method for the determination of residues of Warfarin in soil.
4.2.1	Reliability	1
4.2.2	Deficiencies	None

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
Date **Materials and Methods** **Results and discussion** **Conclusion** **Reliability** **Acceptability** **Remarks**	EVALUATION BY RAPPORTEUR MEMBER STATE
Date **Materials and Methods** **Results and discussion** **Conclusion** **Reliability** **Acceptability** **Remarks**	COMMENTS FROM ...

Table A.4.2-1: Recovery rates for the determination of Warfarin in soil.

Fortification level [mg/kg]	n	Recovery		
		Range[%]	Mean [%] ± RSD	Overall [%] ± RSD
0.02	5	84-97	92 ±6%	95 ±4%
0.2	5	95-98	97 ±1%	

*) limit of quantification;
n: number of determinations;
RSD: relative standard deviation

Section A4.2 Analytical Methods for Detection and Identification in (a) soil

Annex Point IIA4.2

Supportive data

The following data were already submitted in the context of an application for inclusion of the active substance Warfarin in Annex I of Directive 91/414/EEC. Since these data are not considered to represent key studies, they are cited below as supportive data only:

Reference A4.2/02: [redacted] (1984): Analysis method for the determination of Warfarin in the soil by means of HPLC, [redacted] February 13, 1984

Warfarin is determined using high performance liquid chromatography (HPLC) and UV detection. After the initial extraction of the soil sample by acetone and withdrawal of the acetone in a rotary evaporator (40°C), the aqueous residue is acidified with 2 N hydrochloric acid to a pH of 2, and then extracted into chloroform. After removal of the chloroform, the solute is taken up in 0.5 ml of methanol and subjected to HPLC analysis

Cross-Reference IVA 7.2.2.4/01: [redacted] (1999) Aerobic soil degradation of Warfarin [redacted]

This method was developed and validated in connection with studies on aerobic soil degradation, soil photolysis and adsorption/desorption, and in the last step intrinsically involved radio-detection, which is why a full validation for a limit of detection is not reported. The extraction procedure involved acidification of the soil samples, followed by chloroform/1 N HCl extraction, subsequent concentration of the extracts and reversed phase gradient (water - acetonitrile - formic acid) radio-HPLC

Table 4.2-2: Validation data for analytical methods for the determination of residues of Warfarin in soil

Reference (analyte)	Matrix	Fortification level [mg/kg]	Recovery rate [%] mean	range	RSD [%]	n
Kurth, H.-H. (1999)	soil	0.5	>84		no data	2
		5	>84		no data	2
	soil	50	>84		no data	2
Kuhlmann (1984)	soil	n.d.	n.d.	95 – 98	n.d.	n.d.

Section A4.2
Annex Point IIA4.2

Analytical Methods for Detection and Identification in (b) air

1 REFERENCE

Official use only

1.1	**Reference**	**A4.2/03:** [redacted](2002) Validation of an analytical method for the determination of Warfarin from air or airborne Warfarin containing dust (Curattin Haftstreupuder) from air. [redacted] February 01, 2002 (unpublished).
1.2	**Data protection**	Yes
1.2.1	Data owner	Warfarin Task Force
1.2.2	Companies with letter of access	Spiess Urania Chemicals GmbH, Hamburg, Germany; Killgerm Chemicals Ltd., Osset, UK; Hentschke & Sawatzki KG, Neumünster, Germany. Vetyl Chemie GmbH, Illingen, Germany
1.2.3	Criteria for data protection	Data on existing a.s submitted for the first time for entry into Annex I of Directive 98/8/EC.

2 GUIDELINES AND QUALITY ASSURANCE

2.1	**Guideline study**	Yes SANCO/825/00/rev. 6
2.2	**GLP**	Yes
2.3	**Deviations**	None

3 MATERIALS AND METHODS

3.1	**Preliminary treatment**	
3.1.1	Enrichment	Talkum powder (blank-formulation) and Warfarin were spiked on the front filter of the adsorbent tube, filled with Tenax as adsorbent material. Air (humidity > 90%, temperature > 35°C) was drawn through the tube for at least 8 h. Residues were extracted with acetone.
3.1.2	Cleanup	No further clean up was performed, since LC–MS/MS was used for quantification.
3.2	**Detection**	
3.2.1	Separation method	LC–MS/MS (reversed-phase column; mobile phase: acetonitrile/water/acetic acid, gradient)
3.2.2	Detector	LCQ Duo Ion Trap System, electro spray interface (negative polarity), monitored ions: m/z = 308 – > 161.

Section A4.2
Annex Point IIA4.2

Analytical Methods for Detection and Identification in (b) air

3.2.3	Standard(s)	Test substance: Curattin Haftstreupuder, active substance content: 0.5 % Warfarin, batch no.: 012032 Reference substance: Warfarin, batch no.: 10124, purity: 98 %.
3.2.4	Interfering substance(s)	No interfering substances were observed.
3.3	**Linearity**	
3.3.1	Calibration range	The detector response for the analytical standards was found to be linear in the range of 5 ng/ml to 100 ng/ml.
3.3.2	Number of measurements	The calibration curve was plotted based on six different concentrations.
3.3.3	Linearity	The equation of a typical standard calibration function for Warfarin was determined as $y = 133419 + 277420\ x$; $r^2 = 0.9996$ where y is the response in the chromatogram and x the concentration of the substance [ng/ml].
3.4	**Specificity: interfering substances**	The method is suitable for the specific determination of Warfarin in air. No interfering substances were observed. The method is highly specific, since MS/MS was used for detection.
3.5	**Recovery rates at different levels and relative standard deviations**	Please refer to Table A4.2-3.
3.6	**Limit of determination**	The limit of quantification is 0.09 µg/m³.
3.7	**Precision**	
3.7.1	Repeatability	The recovery rates were in a range of 70 – 110 % and the relative standard deviations were below 20 % at each fortification level.
3.7.2	Independent laboratory validation	Not required.

4 APPLICANT'S SUMMARY AND CONCLUSION

4.1	**Materials and methods**	Warfarin and Talkum powder were spiked onto Tenax test tubes and air was subsequently drawn through. Residues were extracted with acetone and determination was performed by LC-MS/MS.
4.2	**Conclusion**	The average recoveries were in a range of 70 – 110 % with relative standard deviations below 20 %. No interfering blanks were observed. Therefore, this method fulfils the requirements of SANCO/825/00 rev.6 as an enforcement method for the determination of residues of Warfarin in air.
4.2.1	Reliability	1
4.2.2	Deficiencies	None

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
Date **Materials and Methods** **Results and discussion** **Conclusion** **Reliability** **Acceptability** **Remarks**	EVALUATION BY RAPPORTEUR MEMBER STATE
Date **Materials and Methods** **Results and discussion** **Conclusion** **Reliability** **Acceptability** **Remarks**	COMMENTS FROM ...

Table A4.2-3: Validation data for the determination of residues of Warfarin in air.

Fortification level [µg/m³]	n	Recovery		
		Range[%]	Mean [%] ± RSD	Overall [%] ± RSD
0.09*	5	67 – 96	83 ± 14%	98 ± 10 %
0.8	5	104 – 112	107 ± 3%	
2.1	5	91 – 99	96 ± 4%	
21.5	5	96 – 105	100 ± 4%	
111	5	92 – 106	100 ± 6%	
410	3	100 – 112	106 ± 6%	

*) limit of quantification;
n: number of determinations;
RSD: relative standard deviation

Section A4.2 Analytical Methods for Detection and Identification in (b) air

Annex Point IIA4.2

Supportive data

The following data were already submitted in the context of an application for inclusion of the active substance Warfarin in Annex I of Directive 91/414/EEC. Since these data are not considered to represent key studies, they are cited below as supportive data only:

Reference A4.2/04: Freitas CM, Levins PL, Smith RH (1980) Warfarin: Measurements research branch analytical method. NIOSH Manual of Analytical Methods 2nd edition, Vol. 6, 313/1-313/8 (published)

A known volume of air is drawn though a polytetrafluoroethylene filter to trap Warfarin, which is subsequently extracted with methanol. Analysis is performed via reversed phase HPLC/UV detection at 280 nm, in an isocratic methanol/phosphoric acid (2.5mM).

Reference A4.2/05: Bagon DA, Warwick CJ (1982) Rationale and methodology of safety of the working environment in the pharmaceutical industry. Safety of Working Environment, 519-522 (published)

Workplace air samples are drawn through a suitable filter at a flow rate of 1 l/min, typical sample volumes: 300-400 litres. Filters are then extracted with 2-5 ml of methanol, subsequent evaporation to dryness and taking up in a reduced methanol volume. Subsequent analysis is via reversed phase HPLC/UV (240 nm) in an isocratic system of acetonitrile/water/acetic acid (750:225:25).

Reference A4.2/06: Arnold JE (1994) Warfarin. Method 5002. NIOSH Manual of Analytical Methods, 4th Ed (published)

Air samples are drawn through a 1-µm PTFE membrane filter at a flow rate of 1-4 l/min, typical sample volumes: 200-1000 litres. Filters are then extracted with 2-5 ml of methanol, subsequent evaporation to dryness and taking up in a reduced methanol volume. Subsequent analysis is via reversed phase HPLC/UV (280 nm) in an isocratic system of methanol/2.5 mM phosphoric acid (70:30).

Table 4.2-4: Validation data for analytical methods for the determination of residues of Warfarin in air

Reference (analyte)	Matrix	Fortification level [mg/m³]	Recovery rate [%] mean	Recovery rate [%] range	RSD [%]	n
Freitas et al. (1980)	air	no data	102.1	no data	no data	no data
Bagon & Warwick (1982)	air	no data	no data	no data	no data	no data
Arnold (1994)	air	0.24	100 %	no data	5.6	no data

Section A4.2 **Analytical Methods for Detection and Identification in (c) water**
Annex Point IIA4.2

Official use only

1 REFERENCE

1.1	**Reference**	**A4.2/07:** [redacted](2001) Residue Analysis of Warfarin in Drinking Water and Surface Water – Method Validation. [redacted] August 17, 2001 (unpublished)
1.2	**Data protection**	Yes
1.2.1	Data owner	Neudorff GmbH, Germany
1.2.2	Companies with letter of access	Spiess Urania Chemicals GmbH, Hamburg, Germany; Killgerm Chemicals Ltd., Osset, UK; Hentschke & Sawatzki KG, Neumünster, Germany. Vetyl Chemie GmbH, Illingen, Germany
1.2.3	Criteria for data protection	Data on existing a.s. submitted for the first time for entry into Annex I of Directive 98/8/EC.

2 GUIDELINES AND QUALITY ASSURANCE

2.1	**Guideline study**	Yes SANCO/825/00/rev. 6
2.2	**GLP**	Yes
2.3	**Deviations**	None

3 MATERIALS AND METHODS

3.1	**Preliminary treatment**	
3.1.1	Enrichment	After acidifying the water samples with acetic acid, residues of Warfarin were extracted on a C_{18} SPE cartridge and eluted with acetonitrile/water/acetic acid (50:50:0.1).
3.1.2	Cleanup	No further clean up was performed, since LC–MS/MS was used for quantification.
3.2	**Detection**	
3.2.1	Separation method	LC–MS/MS (reversed-phase column; mobile phase: acetonitrile/water/acetic acid, gradient)
3.2.2	Detector	LCQ Duo Ion Trap System, electro spray interface (negative polarity), monitored ions: m/z = 308 –> 161.
3.2.3	Standard(s)	Warfarin, batch no. 60205, purity: 99.9 %; water samples: please refer to Table A4.2-5.
3.2.4	Interfering substance(s)	No interfering substances were observed.

Section A4.2 **Analytical Methods for Detection and Identification in (c) water**
Annex Point IIA4.2

3.3	**Linearity**	
3.3.1	Calibration range	The detector response for the analytical standards was found to be linear in the range of 3 ng/ml to 100 ng/ml.
3.3.2	Number of measurements	The calibration curve was plotted based on eight different concentrations.
3.3.3	Linearity	The equation of a typical standard calibration function for Warfarin was determined as $y = 112429 + 132066x - 517.76x^2$; $r^2 = 0.9970$ where y is the response in the chromatogram and x the concentration of the substance [ng/ml].
3.4	**Specificity: interfering substances**	The method is suitable for the specific determination of Warfarin in drinking and surface water. No interfering substances were observed. The method is highly specific, since MS/MS was used for detection.
3.5	**Recovery rates at different levels and relative standard deviations**	Please refer to Table A4.2-6.
3.6	**Limit of quantification**	The limit of quantification is 0.05 µg/l for surface and drinking water.
3.7	**Precision**	
3.7.1	Repeatability	The recovery rates were in a range of 70 – 110 % and the relative standard deviations were below 20 % at each fortification level.
3.7.2	Independent laboratory validation	Not required.

4 APPLICANT'S SUMMARY AND CONCLUSION

4.1	**Materials and methods**	Residues of Warfarin were extracted on a C_{18} SPE cartridge and eluted with a mixture of acetonitrile/water/acetic acid. Determination was performed by LC-MS/MS.
4.2	**Conclusion**	The average recoveries were in a range of 70 – 110 % with relative standard deviations below 20 %. No interfering blanks were observed. Therefore, this method fulfils the requirements of SANCO/825/00 rev. 6 as an enforcement method for the determination of residues of Warfarin in drinking and surface water.
4.2.1	Reliability	1
4.2.2	Deficiencies	None

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
Date **Materials and Methods** **Results and discussion** **Conclusion** **Reliability** **Acceptability** **Remarks**	EVALUATION BY RAPPORTEUR MEMBER STATE
Date **Materials and Methods** **Results and discussion** **Conclusion** **Reliability** **Acceptability** **Remarks**	COMMENTS FROM ...

TableA4.2-5: Water samples: Origin and characteristics.

Parameter	Drinking water	Surface water
Origin	Municipal drinking water supply, Malsch, Germany	River Nagold, sampled at "Werderbrücke", Pforzheim, Germany
Appearance	Colourless and odourless	Colourless and weakly turbid, odourless
pH (at 20°C)	7.3	8.5
Specific electric conductivity (at 20°C, µS/cm)	324	645
Total hardness [mmol/l]	1.6	2.5
Spectral absorption coefficient (at 254 nm, m^{-1})	0.3	4.8
Dissolved organic carbon [mg C/l]	< 0.5	2.3

TableA4.2-6: Recovery rates for the determination of Warfarin in water samples.

Matrix	Fortification level [µg/l]	n	Recovery [%]		
			Range	Mean ± RSD	Overall ± RSD
Drinking water	0.05*	5	84 – 89	86 ± 2%	90 ± 6%
	0.5	5	92 – 98	95 ± 2%	
Surface water	0.05*	5	93 – 99	96 ± 2%	97 ± 4%
	0.5	5	94 – 106	98 ± 5%	

*) limit of quantification; n: number of determinations;
n: number of determinations;
RSD: relative standard deviation

Section A4.2 Analytical Methods for Detection and Identification in (c) water

Annex Point IIA4.2

Supportive data

The following data were already submitted in the context of an application for inclusion of the active substance Warfarin in Annex I of Directive 91/414/EEC. Since these data are not considered to represent key studies, they are cited below as supportive data only:

Reference A4.2/08: Bellar TA, Budde WL (1988) Determination of nonvolatile organic compounds in aqueous environmental samples using liquid chromatography/mass spectrometry. Anal. Chem. **60**, 2076-2083 (published)

Water samples are initially subjected to the following alternative extraction procedures: (I) liquid-liquid extraction: the sample (1 litre) is fortified with 100g/l NaCl, and then extracted (3 times) with methylene chloride, dried over anhydrous sodium sulphate, and after methanol is added, dried to a volume of 1 ml. (II) liquid-solid extraction: 100-ml-samples are bound on a C-18 rev. phase column, and eluted with methanol. Concentrated samples are then subjected to HPLC with MS detection.

Reference A4.2/09: Dalbacke J, Dahlquist I, Persson C (1990) Determination of warfarin in drinking water by high-performance liquid chromatography after solid-phase extraction. J. Chromatogr. **507**, 381-387 (published)

Drinking water (1 litre) samples are acidified with acetic acid, and then solid-phase extracted over bond-elut C-18 (200mg, 3 ml) columns. These are then washed with acetonitrile/water (20:80, pH=4.3), and eluted with acetonitrile/phosphate buffer (1:1, pH=4.3) to 1 ml. Analysis is by HPLC/UV (isocratic system) at 282 or 306 nm.

Reference A4.2/10: Slobodnik J, Brouwer ER, Geerdink RB, Mulder WH, Lingeman H, Brinkman UAT (1992) Fully automated on-line liquid chromatographic separation system for polar pollutants in various types of water. Anal. Chim. Acta **268**, 55-65 (published)

Surface water samples are pre-concentrated with the aid of an automated on-line trace enrichment system: samples of 100-150 ml are solid-phase extracted onto a Prospekt (10mm x 2 mm) cartridge (styrene-divinylbenzene copolymer, 15-25 microns). Desorption is automatically by coupling the cartridge in-line with the analytical column. Analysis is by reversed phase gradient HPLC coupled with diode-array detection.

Reference A4.2/11: Slobodnik J, Groenewegen MGM, Brouwer ER, Lingeman H, Brinkman UAT (1993) Fully automated multi-residue method for trace level monitoring of polar pesticides by liquid chromatography. J. Chromatogr. **642**, 359-370 (published)

Surface water samples are pre-concentrated with the aid of an automated on-line trace enrichment system: samples of 100-150 ml are solid-phase extracted onto a Prospekt (10mm x 2 mm) cartridge (styrene-divinylbenzene copolymer, 15-25 microns). Desorption is automatically by coupling the cartridge in-line with the analytical column. Analysis is by reversed phase gradient HPLC coupled with diode-array detection (the analytical method is identical to reference 4.2/10 above, only during performance some technical details have been adapted).

Table 4.2-7: Validation data for analytical methods for the determination of residues of Warfarin in water (including drinking, ground and surface waters)

Reference (analyte)	Matrix	Fortification level [µg/l]	Recovery rate [%] mean	range	RSD [%]	n
Bellar & Budde (1988)	surface water	10	74	n.d.	10	6
		100	90	n.d.	9	6
Dalbacke et al. (1990)	drinking water	0.05	100	87 – 111	13	3
		0.10	100	96 – 104	4	3
		0.20	99	94 – 104	5	3
Slobodnik et al. (1992)	surface water	5	n.d.	n.d.	0.3	4
Slobodnik et al.(1993)	surface and drinking water	n.d.	n.d.	n.d.	5	8

Section A4.2 **Analytical Methods for Detection and Identification in**
Annex Point IIA4.2 **(d) animal and human body fluids and tissues**

Official use only

1 REFERENCE

1.1	**Reference**	**A4.2/12:** [redacted](2002) Residue analysis of Warfarin in animal tissues and body fluids – method development and validation. [redacted] January 21, 2002 (unpublished).
1.2	**Data protection**	Yes
1.2.1	Data owner	Neudorff GmbH, Germany
1.2.2	Companies with letter of access	Spiess Urania Chemicals GmbH, Hamburg, Germany; Killgerm Chemicals Ltd., Osset, UK; Hentschke & Sawatzki KG, Neumünster, Germany. Vetyl Chemie GmbH, Illingen, Germany
1.2.3	Criteria for data protection	Data on existing a.s submitted for the first time for entry into Annex I of Directive 98/8/EC.

2 GUIDELINES AND QUALITY ASSURANCE

2.1	**Guideline study**	Yes SANCO/825/00/rev. 6
2.2	**GLP**	Yes
2.3	**Deviations**	None

3 MATERIALS AND METHODS

3.1	**Preliminary treatment**	
3.1.1	Enrichment	Residues in milk, meat and eggs were extracted with acetone/water (2:1) followed by partition into cyclohexane/ethyl acetate (1:1) according to the modified multi-residue method DFG S 19. Blood samples were acidified with 1 % acetic acid. Residues were extracted by solid-phase extraction on SPE column and eluted with acetonitrile containing 1 % acetic acid.
3.1.2	Cleanup	Gel permeation chromatography (mobile phase: cyclohexane/ethyl acetate) for milk, meat and eggs.
3.2	**Detection**	
3.2.1	Separation method	LC–MS/MS (reversed-phase column; mobile phase: acetonitrile/water/acetic acid, gradient)
3.2.2	Detector	LCQ Duo Ion Trap System, electro spray interface (negative polarity), monitored ions: m/z = 307 –> 161.
3.2.3	Standard(s)	Warfarin, batch no. 60205, purity: 99.9 %.

Section A4.2
Annex Point IIA4.2

Analytical Methods for Detection and Identification in (d) animal and human body fluids and tissues

3.2.4	Interfering substance(s)	No interfering substances were observed.
3.3	**Linearity**	
3.3.1	Calibration range	The detector response for the analytical standards was found to be linear in the range of 5 ng/ml to 150 ng/ml.
3.3.2	Number of measurements	The calibration curve was plotted based on nine different concentrations.
3.3.3	Linearity	The equation of a typical standard calibration function for Warfarin was determined as $y = -254050 + 2.42105 \times 10^6 x - 6376.64x^2$; $r^2 = 0.9997$ where y is the response in the chromatogram and x the concentration of the substance [ng/ml].
3.4	**Specificity: interfering substances**	The method enables the specific determination of Warfarin in animal tissues and body fluids. No interfering substances were observed. The method is even highly specific, since MS/MS was used for detection.
3.5	**Recovery rates at different levels and relative standard deviations**	Please refer to Table A4.2-8 and Table A4.2-9.
3.6	**Limit of quantification**	The limit of quantification is 0.01 mg/kg for milk, meat and eggs and 0.05 mg/l for blood.
3.7	**Precision**	
3.7.1	Repeatability	The recovery rates were in a range of 70 – 110 % and the relative standard deviations were below 20 % at each fortification level.
3.7.2	Independent laboratory validation	Not required.

4 APPLICANT'S SUMMARY AND CONCLUSION

4.1	**Materials and methods**	Residues in eggs, meat and milk were extracted according to the modified multi-residue method DFG S 19. Residues in blood samples were extracted by solid-phase extraction on SPE column. Determination was performed by LC-MS/MS.
4.2	**Conclusion**	The average recoveries were in a range of 70-110 % with relative standard deviations below 20 %. No interfering blanks were observed. Therefore, this method fulfils the requirements of SANCO/825/00 rev.6 as an enforcement method for the determination of residues of Warfarin in animal tissues and body fluids.
4.2.1	Reliability	1
4.2.2	Deficiencies	None

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
Date **Materials and Methods** **Results and discussion** **Conclusion** **Reliability** **Acceptability** **Remarks**	EVALUATION BY RAPPORTEUR MEMBER STATE
Date **Materials and Methods** **Results and discussion** **Conclusion** **Reliability** **Acceptability** **Remarks**	COMMENTS FROM ...

Table A4.2-8: Recovery rates for the determination of Warfarin in milk, meat and eggs.

Matrix	Fortification level [mg/kg]	n	Recovery		
			Range[%]	Mean [%] ± RSD	Overall [%] ± RSD
Milk	0.01*	5	69 – 96	82 ± 12%	82 ± 8%
	0.1	5	77 – 88	82 ± 5%	
Meat	0.01*	5	80 – 86	82 ± 3%	82 ± 3%
	0.1	5	79 – 85	81 ± 3%	
Eggs	0.01*	5	75 – 87	80 ± 7%	80 ± 7%
	0.1	5	75 – 88	81 ± 7%	

*) limit of quantification
n: number of determinations
RSD: relative standard deviation

TableA4.2-9: Recovery rates for the determination of Warfarin in blood.

Fortification level [mg/l]	n	Recovery		
		Range [%]	Mean [%] ± RSD	Overall [%] ± RSD
0.05*	5	86 – 98	90 ± 6%	92 ± 6%
0.5	5	84 – 98	93 ± 6%	

*) limit of quantification
n: number of determinations
RSD: relative standard deviation

Section A4.2 Analytical Methods for Detection and Identification in
Annex Point IIA4.2 (d) animal and human body fluids and tissues

Supportive data

The following data were already submitted in the context of an application for inclusion of the active substance Warfarin in Annex I of Directive 91/414/EEC. Since these data are not considered to represent key studies, they are cited below as supportive data only:

Reference A4.2/13: Fasco MJ, Cashin MJ, Kaminsky LS (1979) A novel method for the quantitation of Warfarin and its metabolites in plasma. J. Liq. Chromatogr. **2**, 565-575 (published)

This method allows quantitation of Warfarin and its main metabolites (4-, 6-, 7-, 8- Warfarin alcohols, and dehydrowarfarin) in human plasma. Plasma samples are acidified by mixing 1:1 with ammonium acetate (pH=4.85) and adsorbed onto Sep-Pak C-18, which were washed free of polar constituents with ammonium acetate buffer, pH=4.5 and dried prior to elution with methanol. The eluate is taken up in mobile phase and analysed via reversed phase (acetonitrile gradient) HPLC/UV (313 nm).

Reference A4.2/14: Wong LT, Solomonraj G, Thomas BH (1977) Analysis of Warfarin in plasma by high-pressure liquid chromatography. J. Chromatogr. **135**, 149-154 (published)

Human plasma samples (1-2 ml) are acidified by addition of phosphate buffer and 3N HCl, and extracted with methylene chloride (internal standard: methylated Warfarin). After drying, the residue is re-dissolved in dioxane, and analysed via HPLC/UV in a mobile phase of 40% dioxane in water (pH=4.2).

Reference A4.2/15: Naidong W, Lee JW (1993) Development and validation of a high-performance liquid chromatographic method for the quantitation of Warfarin enantiomers in human plasma. J. Pharm. Biomed. Anal. **11**, 785-792 (published)

This very sensitive and well validated method allows the separate quantitation of the R- and S-enantiomers of Warfarin in human plasma. Plasma samples (1 ml) are acidified with 1 N sulphuric acid, and after mixing are extracted with ethyl ether. After drying, the extract is reconstituted in acetonitrile. Analysis is by reversed phase HPLC/UV (320 nm) on a stereoselective β-cyclodextrine column using a mobile phase of acetonitrile/acetic acid/TEA (1000:3:2.5).

Reference A4.2/16: Felice LJ, Chalermchaikit T, Murphy MJ (1991) Multicomponent determination of 4-hydroxycoumarin anticoagulant rodenticides in blood serum by liquid chromatography with fluorescence detection. J. Anal. Toxicol. **15**, 126-129 (published)

This method was developed to enable the analysis of several anticoagulant rodenticides in blood serum. Initially, blood samples are mixed with acetonitrile and vortexed to precipitate blood proteins, followed by centrifugation. Particularly in the case of Warfarin, the recovery is enhanced if the precipitate is washed again with acetonitrile. The supernatant is then extracted with ethyl ether to remove residual water and any polar material, and to reduce evaporation time. The residue is then first dissolved in methanol, and mobile phase buffer is added. Analysis is by reversed phase gradient (ammonium acetate-methanol) HPLC with fluorescence detection (excitation 318 nm, emission 390 nm).

Reference A4.2/17: De Wolff FA, Tetteroo-Tempelman CAM, Edelbroek PM (1980) Determination of nanogram levels of the anticoagulant acenocoumarin in serum by high-performance liquid chromatography. J. Anal. Toxicol. **4**, 156-159 (published)

Internal standard (5-methoxypsoralen) is added to 1-ml serum or plasma samples, followed by 1 ml of sodium acetate buffer (pH=4.0). After mixing, a liquid-liquid extraction is performed by adding petroleum ether/dichloromethane (1:1). The organic phase is separated, evaporated to dryness under a stream of nitrogen, and re-dissolved in mobile phase (acetonitrile/0.1% acetic acid, 35:65). Analysis is by isocratic reversed phase HPLC with UV detection (308 nm).

Reference A4.2/18: Park SW, Seo B, Kim E, Kim D, Paeng K-J (1996) Purification and determination procedure of coumarin derivatives. J. For. Sci. **41**, 685-688 (published)

Animal tissue (rat organs: heart, lung, liver kidney) and blood sample are homogenised , acidified to pH 3-4 with sulphuric acid, and extracted three times with 10% methanol in chloroform. Extracts are then evaporated to dryness and re-dissolved in cyclohexane. This extract is then further purified over Florisil, bound onto Sep-Pak cartridges and finally eluted with methanol. Analysis is by isocratic (methanol/0.8% acetic acid, 8:2) reversed phase HPLC with diode array detection.

Reference A4.2/19: Bergmann A, Kruzik P, Weiser M (1985) Analytical procedures for identification of the rat poisons Racumin, Tomorin and Warfarin in biological material. Wien. tieraerztl. Mschr. **72**, 250-254 (published)

Samples are acidified with 3 M HCl and extracted with dichloromethane. The extract is evaporated to dryness, re-dissolved in mobile phase (acetonitrile/ammonium acetate pH = 4.5, 1:1). Analysis is by isocratic reversed phase HPLC with UV detection (313 nm).

Reference A4.2/20: Hunter K (1985) High-performance liquid chromatographic strategies for the determination and confirmation of anticoagulant rodenticide residues in animal tissues. J. Chromatogr. **321**, 255-272 (published)

Animal tissue samples are chopped, dried by admixture of anhydrous sodium sulphate and homogenised in chloroform/acetone (1:1). The extract is the filtered, residual material re-extracted, and these extracts are pooled with the washings of the homogeniser. This filtrate is then evaporated to dryness and re-dissolved in hexane/chloroform/acetone (75:20:5). The subsequent clean-up step involves a gel permeation chromatography, followed by adsorption onto an on-line Sep-Pak cartridge, from which Warfarin is eluted with 0.25 formic acid in dichloromethane. Analysis is by reversed phase gradient (methanol/water, both containing 0.25% acetic acid) HPLC with UV detection (254 or 280 nm).

Reference A4.2/21: Berny PJ, Buronfosse T, Lorgue G (1995)Anticoagulant poisoning in animals: A simple new high-performance thin-layer chromatographic (HPTLC) method for the simultaneous determination of eight anticoagulant rodenticides in liver samples. J. Anal. Toxicol. **19**, 576-580 (published)

This method covers the analysis of several anticoagulant rodenticides in animal tissues. Liver or plasma samples were mixed with acetone, homogenised and then centrifuged. The supernatant was separated, and the residue extracted once more with acetone. The supernatant fractions were combined, and di-ethylether was added for precipitation of proteins. After centrifugation, the extracts are evaporated to dryness and re-dissolved in methanol. Application to the TLC plates (nano HPTLC RP18) is then performed by an automatic TLC sampler, followed by development in a system of methanol/4.7 M phosphoric acid (9:1). Quantitation was performed using a TLC scanner.

Reference A4.2/22: Jones A (1996) HPLC determination of anticoagulant rodenticide residues in animal livers. Bull. Environ. Contam. Toxicol. **56**, 8-15 (published)

Tissue samples were cut and ground with 10 x sample weight of sodium sulphate in a mortar, and subsequently extracted twice with 30% acetone in dichloromethane. The extracts were centrifuged, the supernatants were further adsorbed onto a Sep-Pak cartridge, and eluted with 5% acetic acid in methanol. The eluate was evaporated to dryness, and re-dissolved in methanol. Analysis was by reversed phase gradient (methanol/0.25% acetic acid) HPLC with fluorescence detection (excitation 310 nm, emission 390 nm).

Reference A4.2/23: Gaillard Y, Pepin G (1997) Screening and identification of drugs in human hair by high-performance liquid chromatography-photodiode-array UV detection and gas chromatography-mass spectrometry after solid-phase extraction. A powerful tool in forensic medicine. J. Chromatogr. A **762**, 251-267 (published)

This system represents a forensic screening method for identification of drugs including Warfarin in human hair. A minute sample of hair (75 mg) is extracted at 56°C in water, concentrated by solid-phase extraction at pH = 2 and eluted with 1 % ammoniacal methanol. Analysis is by reversed phase HPLC and photodiode array detection.

Reference A4.2/24: Langseth W, Nymoen U (1991) Determination of coumarin anticoagulant rodenticide residues in animal liver by high-performance liquid chromatography. Fres. J. Anal. Chem. **339**, 249-252 (published)

Tissue (liver) samples are homogenised in ethanol and centrifuged. The supernatant is evaporated to dryness, and the residue then re-dissolved in toluene. To this, hexane is added 1:1, plus anhydrous sodium sulphate. The solute is then adsorbed onto a silica Bond Elut column, and eluted with toluene/methanol (97:3). The final eluate is dried and re-dissolved in methanol. Analysis is by reversed phase isocratic (methanol/35 mM acetic acid, 75:25) HPLC with fluorescence detection (excitation 308 nm, emission 380 nm).

Reference A4.2/25: Chalermchaikit T, Felice LJ, Murphy MJ (1993) Simultaneous determination of eight anticoagulant rodenticides in blood, serum and liver. J. Anal. Toxicol. **17**, 56-61 (published)

Serum samples are mixed with acetonitrile for protein precipitation, vortexed and centrifuged. The residue is extracted again, the extract centrifuged, the combined extracts passed through a florisil/aluminium oxide column, and then evaporated to dryness. The residue is re-dissolved in methanol. Liver samples are homogenised twice in acetonitrile, the extracts centrifuged, the supernatant passed through a florisil/aluminium oxide column, and then evaporated to dryness. The residue is re-dissolved in methanol. Analysis is by reversed phase gradient (methanol/ammonium acetate) HPLC with fluorescence detection (excitation 318 nm, emission 390 nm).

Reference A4.2/26: Lang D, Böcker R (1995) Highly sensitive and specific high performance liquid chromatographic analysis of 7-hydroxywarfarin, a marker for human cytochrome P4502C9 activity. J. Chromatogr. B **672**, 305-309 (published)

This method covers the analysis of Warfarin and three metabolites from human liver extracts. The Warfarin metabolism in the human liver extracts was stopped by quenching with 10 µl of perchlorid acid and 7-ethoxycoumarin was added as an internal standard. Denaturised proteins were precipitated by centrifugation at 3000 g for 5 min (4 °C) and 100 µl of the supernatant was used for HPLC analysis. Analysis was performed by reversed phase isocratic (acetonitrile/0.5 % phosphoric acid, 38:62 v/v, 1.3 ml/min) HPLC with UV (205 nm) and fluorescence detection (excitation 320 nm, emission 415 nm).

Table A4.2-10: Validation data for analytical methods for the determination of residues of Warfarin in body fluids and tissues of animal and human origin

Reference (analyte)	Matrix	Fortification level [µg/ml]	Recovery rate [%] mean	range	RSD [%]	n
Fasco et al (1979)	human plasma	0.5 – 10.0	>95	no data	no data	no data
Wong et al. (1977)	human plasma	1.25	98.4	no data	1.6	3
		2.5	88.0	no data	4.5	3
		5.0	90.2	no data	5.3	3
Naidong & Lee (1993)	human plasma	0.0125	84	no data	9.4	3
		0.250	73.4	no data	1.7	3
		2.5	78.2	no data	3.6	3
Felice et al. (1991)	blood serum	0.05	68.1	no data	3.0	8
		0.25	79.8	no data	3.0	7
De Wolff et al. (1980)	human serum	no data	no data	no data	no data	no data
Park et al. (1996)	animal tissue	5	97	96 – 98	2	6
Bergmann et al. (1985)	animal tissue	no data	no data	no data	no data	no data
Hunter (1985)	animal tissue	0.05 mg/kg	95	no data	4.2	3
		0.2 mg/kg	98	no data	2.6	3
		1 mg/kg	97	no data	3.2	3
Berny et al. (1995)	animal tissue	no data	87.5	no data	< 5	3
Jones (1996)	animal tissue	0.04 mg/kg	96	no data	7.6	6
		0.2 mg/kg	91	no data	6.8	6
		1 mg/kg	96	no data	6.0	6
Gaillard & Pepin (1997)	human hair	10 mg/kg	81.2	no data	5.3	10
Langseth & Nymoen (1991)	animal tissue	0.5 mg/kg	80	75 – 85	no data	no data
Chalermchaikit et al. (1993)	animal tissue and serum	50 ng/ml serum	96	no data	3.2	no data
		250 ng/ml serum	98.5	no data	3.2	no data
		50 mg/kg	81.9	no data	7.2	no data
		250 mg/kg	86.4	no data	3.1	no data

Table A4.2-11: Validation data for analytical method for the determination of Warfarin and metabolites in human liver extracts.

Reference	**Matrix**	**Substance**	**Detection limit**	**Reproducibility**	**Recovery (%)**
Lange & Böcker (1995)	Human liver extract	Warfarin	no data	no data	no data
		7-Hydroxywarfarin	150 fmol	1.11 % (SD) at 6.2 pmol	92.7 ± 0.8 % 30.8 – 2.5 µM
		6-Hydroxywarfarin	5 pmol	no data	no data
		4´-Hydroxywarfarin	3 nmol	no data	no data

Section A4.3
Annex Point IIIA 6.1

Analytical methods including recovery rates and the limits of determination of the active substance, and for residues thereof, in/on food or feedstuffs and other products where relevant.

	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [ ]	**Technically not feasible [ ]** **Scientifically unjustified [ ]**	
Limited exposure [X]	**Other justification [ ]**	
Detailed justification:	An analytical determination of residues in/on food or feedstuff is only required if the active substance or the material treated with it is to be used in a manner which may cause contact with food or feedstuff or intended to be placed on, in or near soils in agricultural or horticultural use. The recommendations for use of anticoagulant rodenticides such as Warfarin clearly precludes any such contamination, which is why the submission of such methods is not considered to be necessary.	
Undertaking of intended data submission [ ]		

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
Date **Evaluation of applicant's justification** **Conclusion** **Remarks**	EVALUATION BY RAPPORTEUR MEMBER STATE (*)
Date **Evaluation of applicant's justification** **Conclusion** **Remarks**	COMMENTS FROM ...

Section A5 (Annex Point) — Effectiveness against target organisms and intended uses

			Official use only
5.1	**Function (IIA5.1)**	Rodenticide PT 14	
5.2	**Organism(s) to be controlled and products, organisms or objects to be protected (IIA5.2)**		
5.2.1	Organism(s) to be controlled	The primary target (harmful) organisms for which Warfarin is submitted for registration are the Brown rat (*Rattus norvegicus*), Black rat (*Rattus rattus*) and House mouse (*Mus musculus*). Warfarin is in use world-wide for the control of various commensal and field rodents as well as for the control of other species. In Table 5-1, an effort has been made to summarise the existing data from published sources concerning the susceptibility of harmful organisms to Warfarin.	
5.2.2	Products, organisms or objects to be protected	Warfarin and its products are registered in the form of ready-to-use baits for the control of commensal rodents (rats and mice) in open areas, in and around buildings, sewage systems and landfill sites.	
5.3	**Effects on target organisms, and likely concentration at which the active substance will be used (IIA5.3)**	Warfarin acts as an anticoagulant, with the target rodent species (rat and mice) being particularly sensitive to (ultimately fatal) internal bleeding and haemorrhaging. The rodents ingest Warfarin via consumption of poisoned baits. Nature of the effect: anticoagulation after ingestion	
5.3.1	Effects on target organisms	The purpose of rodenticides is to control rodent pests by killing the animals. Warfarin is a first-generation anticoagulant that is highly toxic to mammals on repeated ingestion, but of lesser, variable toxicity to different species when given as a single oral dose. Studies that specifically deal with related aspects of "effectiveness", e.g. susceptibility of Warfarin-resistant strains, median effective doses etc. are reported in Table A5-1.	
5.3.2	Likely concentrations at which the A.S. will be used	Concentration of active substance in ready-to-use baits is: in wax blocks up to 0.08 % (m/m) ≡ 800 mg/kg in granular bait up to 0.08 % (m/m) ≡ 800 mg/kg	
5.4	**Mode of action (including time delay) (IIA5.4)**	**References:** **A5.4/01:** Thijssen HHW (1995) Warfarin-based rodenticides: mode of action and mechanism of resistance. Pestic. Sci. **43**: 73–78 (published). **A5.4/02:** Olson RE (1984) The function and metabolism of vitamin K. Ann. Rev. Nutr. **4**: 281–337 (published). **A5.4/03:** Fasco MJ, Principes LM (1982) R- and S-Warfarin inhibition of vitamin K and vitamin K 2,3-epoxide reductase activities in the rat. J. Biol. Chem. **257**: 4894-4901 (published).	

Section A5 (Annex Point) — Effectiveness against target organisms and intended uses

			Official use only
5.4.1	Mode of action	Warfarin and Warfarin Sodium both act as anticoagulants that are highly toxic to mammals on repeated ingestion, but of lesser, variable toxicity to different species when given as a single oral dose. In brief, Warfarin inhibits the formation of prothrombin by replacing Vitamin K in the complex, and this reduces the clotting capacity of blood, as well as increasing capillary permeability. This mode of action (of Warfarin, and related anticoagulant rodenticides) depends on the inhibition of the vitamin-K dependant carboxylation of glutamic acid residues in blood clotting factors II (prothrombin), VII, IX and X. In the carboxylase reaction, vitamin K is oxidised to vitamin K 2,3-epoxide (vitamin KO). This epoxide is recycled to the hydroquinone in two steps by the enzyme "vitamin KO reductase" (Thijssen, 1995: reference A5.4.1/01; Olson, 1984: reference A5.4.1/02). As demonstrated in Warfarin-sensitive rats, the anticoagulant binds tightly, and essentially irreversibly to the vitamin KO reductase, thus inhibiting the enzyme and interrupting the cellular recycling of vitamin K. (Fasco, 1982: reference A5.4.1/03; Thijssen, 1987: cross-reference A5.7/12). An alternative pathway performed by a Warfarin-insensitive, but low affinity NAD(P)H-dependant vitamin K reductase needs high vitamin K levels, and is therefore only of importance at pharmacological doses of vitamin K. Vitamin K, when given as an antidote, is thought to act via this pathway.	
5.4.2	Time delay	A time delay is an intrinsic property of all coumarin-like anticoagulants as a consequence of their mode of action: As the hepatic vitamin K cycle is disrupted, adverse effects occur after vitamin K deposits are depleted and, as a consequence, internal haemorrhages occur.	
5.5	**Field of use envisaged (IIA5.5)**	MG03: Pest control Product types PT14	
5.6	**User (IIA5.6)**		
	Industrial	not relevant	
	Professional	Professionally trained users (i.e., pest control operators) may be exposed during application of ready-to-use baits (wax blocks or grain baits). Handling includes either fixing the wax blocks to protected baiting stations or applying the grain bait to specially designed bait boxes. Exposure is predominantly dermal.	
	General public	Primary exposure is considered to be either "zero", or only incidental, i.e. in rare occasions of placing ready-to-use bait packages for home pest control. Exposure is considered to be minimal, since the product will be provided in ready-to-use packaged bait stations (boxes) which will not require the untrained user to handle the bait material directly in any way. Secondary exposure is deemed negligible for the reasons set forth above.	

Section A5 (Annex Point) — Effectiveness against target organisms and intended uses

Official use only

5.7 Information on the occurrence or possible occurrence of the development of resistance and appropriate management strategies (IIA5.7)

Warfarin was introduced as a rodenticide in the late 1940s. Its ideal rodenticide properties have enhanced its employment world-wide. The first inheritable form of Warfarin resistance was discovered approximately 20 years after introduction in wild rats of the strain *Rattus norvegicus* in Scotland (Boyle, 1960: reference A5.7/02). In the following years, rare cases of resistance in rats to Warfarin (and other anticoagulant rodenticides) were reported for two other parts of Great Britain (Greaves, 1970: reference A5.7/06), for distinct areas in North America (Jackson, 1995: reference A5.7/04; Misenheimer, 1990: reference A5.7/05), and Europe (Myllymaeki, A., 1995: reference A5.7/03; Pelz, 1995: reference A5.7/01). A summary is given in Table A5-2.

5.7.1 Development of resistance

References:

A5.7/07: Greaves JH, Ayres P (1967) Heritable resistance to warfarin in rats. Nature (London) **215:** 877-878 (published).

A5.7/08: Partridge GG (1980) The vitamin K requirements of wild brown rats (*Rattus norvegicus*) resistant to warfarin. Comp. Biochem. Physiol. **66A**: 83-87(published).

A5.7/09: Thijssen HHW, Janssen CAT, Mosterd JJ (1989) Warfarin resistance: biochemical evaluation of a warfarin-resistant wild brown rat. Biochem. Pharm. **38:** 3129-3132 (published).

A5.7/10: Whitlon DS, Sadowski JA, Suttie JW (1978) Mechanism of coumarin action: significance of vitamin K epoxide reductase inhibition. Biochemistry **17:** 1371-1379 (published).

A5.7/11: Hildebrandt EF, Suttie JW (1982) Mechanism of coumarin action: Sensitivity of vitamin K metabolizing enzymes of normal and warfarin-resistant rat liver. Biochemistry **21:** 2406-2411 (published).

A5.7/12: Thijssen HHW (1987) Warfarin resistance: Vitamin K epoxide reductase of Scottish resistance gene is not irreversibly blocked by Warfarin. Biochem. Pharmacol. **36:** 2753-2757 (published).

A5.7/13: MacNicoll AD (1995) A review of biochemical mechanisms of warfarin resistance in the house mouse. Pestic. Sci, **43:** 57-59 (published).

The resistance to Warfarin is inheritable: in rats, the phenotypic expression appears to be conducted to a single autosomal gene (Greaves, 1967: reference A5.7/07; Partridge, 1980: reference A5.7/08). At present, there are two distinct Warfarin resistance genotypes, the Welsh and the Scottish, characterised by a clear difference in biochemistry of vitamin K epoxide reductase. A third genotype has been reported from Chicago (Misenheimer, 1990: reference A5.7/05) and is in discussion for differences to the Welsh and the Scottish.

Warfarin-resistant brown rats living in the eastern parts of The Netherlands were demonstrated to be of the Welsh type (Thijssen, 1989: reference A5.7/09). A comprehensive review of the resistance mechanism is given by Thijssen (1995: cross-reference A5.4/01).

As evaluated in point 5.4.1, the mode of action of Warfarin (and related anticoagulant rodenticides), depends on the inhibition of the vitamin KO reductase, presumable by binding of Warfarin to the active centre of the enzyme. In the Welsh-type resistant rat strain, the vitamin KO reductase activity is much less sensitive to Warfarin inhibition than in normal rats (Whitlon, 1978: reference A5.7/10; Hildebrandt, 1982: reference A5.7/11). The biochemical basis lies in an altered enzyme expressing

Section A5 (Annex Point) **Effectiveness against target organisms and intended uses**

			Official use only
		reduced reactivity to Warfarin. Due to this mode of resistance, rats of the Welsh type need an enhanced dietary intake of Vitamin K. Rats of the Scottish phenotype differ from the Welsh strain by their lower level of resistance to Warfarin, and by the absence of a higher requirement for dietary vitamin K. In vitro studies show that the vitamin KO reductase of these rats is as sensitive to Warfarin as the normal enzyme (Thijssen, 1987: reference A5.7/12). In contrast to the normal enzyme, however, the interaction with Warfarin was found to be reversible, so that, although complexed with coumarin, the enzyme remains sensitive for vitamin KO reduction. In house mice, the resistance in some strains depends on a reduction in sensitivity of hepatic vitamin KO reductase to inhibition by Warfarin which is similar to the mechanism evaluated for Warfarin resistant rats. However, there is evidence that other resistance mechanisms may exist in some mice populations instead or in addition to the resistance via KO reductase modification. Detoxification enzymes (cytochrome P-450 with enhanced activity, for example) may be involved in more rapid metabolism of Warfarin and by this mode of action in reduced susceptibility to Warfarin (MacNicoll, 1995: reference A5.7/13).	
5.7.2	Management strategies	**Reference:** **A5.7/14:** Greaves JH (1995) Managing resistance to anticoagulant rodenticides: an appraisal. Pestic. Sci. **43:** 79-82 (published). The use of Warfarin and other anticoagulant rodenticides has resulted in some cases in the selection of Warfarin-resistant rat and mouse strains at various geographical locations. However, these resistant rodent strains are restricted to distinct areas, so that rat populations in most areas are still well controlled by Warfarin. For example, in Great Britain in 1970, there were only 3 areas of 800 miles^2 (Scotland), of 1000 miles^2 (Montgomeryshire and Shropshire) and of 40 – 50 miles^2 (Kent), respectively, affected by resistant rat populations (Greaves, 1970: reference A5.7/06). In north-west Germany near the border to the Netherlands (between the cities Lingen, Osnabrück and Münster), populations of rats exist which are resistant to Warfarin, Bromadiolone and Difenacoum (Pelz, 1995: reference A5.7/01). Apart from these areas, there have been no further detailed reports on cases of resistance. Resistance to Warfarin is inheritable which may enable effective strategies for resistance management to prevent an outspread of resistant populations (Greaves, 1995: reference A5.7/14). The following strategy to avoid and in the case of failure to manage resistance can be proposed: - consequent application of poisoned baits and/or tracking powder until total extermination of the rats, accompanied by sealing former hiding places as well as constructional and other preventive precautions. - continuous monitoring of wild rats for resistance to Warfarin and other anticoagulants within the rodent controlled area. - in case of detection of resistance, assessment of the area within which resistant populations have spread out including a "safety zone" (Greaves stated that prompt and sustained control programs within a 20 km radius surrounding the resistance focus should very likely extinguish the majority of resistant populations). - prompt an effective elimination of resistant populations within the defined area, using second-generation anticoagulants and/or alternative rodenticides after verification that resistant rat strains are susceptible to these poisons.	

Section A5 (Annex Point) — Effectiveness against target organisms and intended uses

			Official use only
5.8	**Mode of action (including time delay) in so far as not covered by section A5.4 (IIB5.8)**	No further information in surplus to that already covered under 5.4 above is available. Considering the well-established anticoagulant action, any further such information is also not considered to be required.	
5.9	**Likely tonnage to be placed on the market per year (IIA5.8)**	Based on the notifications submitted by all applicants, the likely tonnage of Warfarin in biocidal products (rodenticides) currently placed on the market is approx. 0.2 tonnes a.s. per year, without any tendency to increase or decrease in the near future.	

Table A5-1: Summary table of experimental data on the effectiveness of Warfarin against target organisms (rodents). Generally, the substance functions as a rodenticide (PT 14) and the envisaged field of use is pest control (MG03).

Test substance	Test organism(s)	Test method	Test conditions	Test results: effects, mode of action, resistance	Reference
Warfarin	Grey squirrel (*Sciurus carolinensis*) were trapped in various woods and accustomed to the cages for at least three weeks	Chronic toxicity	four squirrels per cage application: oral by gavage, daily (up to 26 days); dose: 0.01, 0.5, 1.25, 6.0 mg/kg bw observation: prothrombin time (PT), mortality.	Animals treated with Warfarin showed an elevated PT even at the lowest dose at day 7. There was high mortality in all groups with the exception of the lowest dose group. One animal of the 0.5 mg/kg dose level (the only survivor in this group) was found to be more tolerant to Warfarin as compared to the others.	**A5.2/01:** Chambers CM, Chambers PL (1983) Warfarin and the grey squirrel. Arch. Tox. Suppl. **6**: 214-221 (published)
Warfarin	Golden hamster (*Mesocricetus auratus*), wild caught or obtained commercial	No-choice feeding tests with poisoned baits	singly caged dose: 0.025, 0.25, 0.5% Warfarin, application period: 28 days, 56 days (highest dose level)	No mortality was observed in both lower dose groups. Even with baits containing 0.5% Warfarin, only 75% of the animals died (days 26-29). The results indicate exceptional resistance to Warfarin.	**A5.2/02:** Bradfield AAG, Gill JE (1984) Laboratory trials of five rodenticides for the control of *Mesocricetus auratus* Waterhouse. J. Hyg. **93:** 389-394 (published)
Warfarin	Cotton rat (*Sigmodon hispidus*)	No-choice feeding tests	application: 0.025% Warfarin baits period: 6 days	100% mortality was observed after 6 days lethal feeding period (with 95 % fiducial limits) LFP_{50}: 3.7 (2.8 – 4.3); LFP_{98}: 7.4 (5.7 – 19.2)	**A5.2/03:** Gill JE, Redfern R (1980) Laboratory trials of seven rodenticides for use against the cotton rat (*Sigmodon hispidus*). J. Hyg. **85:** 443-450 (published)
Warfarin	Norway rat (*Rattus norvegicus*)	No-choice feeding test	0.005% Warfarin baits; animals: collected from several places of Japan	Most rats died within approximately 5 days after initiation of the Warfarin feeding. However, there was some evidence of Warfarin resistance in one rat population (trapped in Yumenoshima).	**A5.2/04:** Taniguchi N, Kato T, Ikeda Y (1985) Rodenticidal activity of warfarin against wild Norway rat *Rattus norvegicus*, collected from some locations in Japan. Jpn. J. Sanit. Zool. **36:** 107-110 (published)

Test substance	Test organism(s)	Test method	Test conditions	Test results: effects, mode of action, resistance	Reference
Warfarin	house mouse (*Mus musculus*)	Field trials (pallet and maize field)	exposure: daily (day 1-7 and day 16-22); dose: 50 g per baiting point, 12 baiting points observation: bait consumption and reopening of closed mouse holes	After 7 days of administration a reduction of infestation was achieved. Due to immigration from the surrounding areas a new infestation was present after additional 8 days.	**A5.2/05:** Bäumler W, Asran AA (1987) Susceptibility of house mice (*Mus musculus*) of different origin to anticoagulants. Anz. Schädlingsk., Pflanzenschutz, Umweltschutz **60:** 1-6 (published)
Warfarin	Nil rat (*Arvicanthis niloticus*) from a laboratory breeding colony	No-choice and choice feeding tests	0.025% Warfarin baits (and unpoisoned baits, respectively).	Warfarin was toxic to all animals (100% mortality after 6 days). lethal feeding period LFP_{50}: 3.8; LFP_{98}: 5.8	**A5.2/06:** Gill JE, Redfern R (1977) Some laboratory tests of five rodenticides for the control of Arvicanthis niloticus. PANS **23**: 33-37 (published)
Warfarin	Egyptian spiny mouse (*Acomys cahirinus*) were live-trapped and accustomed to the cages for 7 days	No-choice feeding tests	Application: 0.025% Warfarin baits; period: up to 28 days.	The spiny mouse was found to be very resistant to Warfarin (for example, only 50% mortality was observed after 28 days of feeding).	**A5.2/07:** Mahmoud W, Redfern R (1981) The response of the Egyptian spiny mouse (*Acomys cahirinus*) and two other species of commensal rodents to anticoagulant rodenticides. J. Hyg. **86**: 329-334 (published)
Warfarin (technical grade from Sorex Ltd.)	Gerbil (*Meriones shawi*) from a laboratory breeding colony	No-choice and choice feeding tests	0.025% Warfarin baits (and unpoisoned baits, respectively).	The gerbil was found to be less susceptible to Warfarin (10% mortality after 28 days no-choice feeding)	**A5.2/08:** Gill JE, Redfern R (1983) Laboratory tests of seven rodenticides for the control of *Meriones shawi*. J. Hyg. **91:** 351-357 (published)

Test substance	Test organism(s)	Test method	Test conditions	Test results: effects, mode of action, resistance	Reference
Warfarin	Field mouse (*Mus booduga*) were trapped from fields and grassland in India.	No-choice feeding test	dose level: 0.25 g/kg bw Warfarin; feeding period: 8 days.	Warfarin was found to be less toxic as compared to second generation anticoagulants Nevertheless, Warfarin was toxic to all animals (100% mortality after 8 days).	**A5.2/09:** Balasubramanyam M, Purushotham KR (1988) The susceptibility of the indian field mouse *Mus-booduga* Gray to anticoagulant rodenticidal baits. Pestic. Sci. **23:** 209-213 (published)
Warfarin	Field mouse (*Mus booduga*) were trapped from fields and grassland in India.	No-choice feeding tests	dose levels: 0.0125%, 0.025, 0.05% application period: 6 days. observation: 21 days post-exposure period	With 0.025% Warfarin (the concentration normally used for rodent control), 83% of the animals had died after 6 days feeding.	**A5.2/10:** Balasubramanyam M, Christopher MJ, Purushotham KR (1984) Laboratory trials of three anticoagulant rodenticides for use against the Indian field mouse. *Mus booduga* Gray; J. Hyg. **93:** 575-578 (published)
Warfarin	Indian crested porcupine (*Hystrix indica*)	Field trial		Warfarin was found less effective against the porcupine as compared to other poisons tested (Compound 1080 and temik for example).	**A5.2/11:** Arshad MI, Khan RA, Khaliq A (1988) Strategies for the control of indian crested porcupine, *Hystrix-indica*. Pak. J. Sci. Ind. Res. **31:** 784-785 (published)
Warfarin (technical grade, 99.6 %)	Gerbil (*Tatera indica*), live trapped near crop fields in India	(i) short time toxicity (ii) feeding tests	(i) application: oral by gavage, 10 – 60 mg/kg at 4 days (ii) 0.025% Warfarin baits (7 days).	(i) 100 % mortality at 60 mg/kg (ii) 100% mortality after 7 days exposure to the Warfarin baits (no-choice feeding). lethal feeding period (with 95 % fiducial limits) LFP_{50}: 4.36 (3.55 – 5.37); LFP_{98}: 6.53 (4.79 – 8.91)	**A5.2/12:** Balasubramanyam M, Shobarani D, Maddaiah GP, Purushotham KR. (1988) Responses to Warfarin by the Indian gerbil *Tatera indica* from Tirupati, India. Indian J. Exp. Biol. **26:** 694-696 (published)

Test substance	Test organism(s)	Test method	Test conditions	Test results: effects, mode of action, resistance	Reference
Warfarin	Various rodent species (rats, mice, squirrels, gerbils)	Field trial, house complexes of Indian desert	0.025% Warfarin baits; baiting period: 15 days.	Warfarin was found to be efficient to most of the rodents.	**A5.2/13:** Advani R, Prakash I (1987) Variations in the rodent populations in response to four anticoagulant rodenticides in the residential habitat of the Indian desert. Pesticides **21:** 18-22 (published)
Warfarin (technical grade from Rentokil Pty Ltd)	Feral pigs (*Sus scrofa*) were trapped in Australia and accustomed to the cages	Acute toxicity	application: oral by bait feeding; dose: (i) 8.3 – 17.3 mg/kg bw (single dose), (ii) 1.3 – 6.8 mg/kg bw (2 doses at two consecutive days).	The following LD were obtained: (i) LD90 > 20 mg/kg bw (ii) LD50 = 2.9 mg/kg bw, LD90 = 6.1 mg/kg bw Additionally, in a choice-feeding test, baits with Warfarin were found to be highly acceptable to feral pigs.	**A5.2/14:** O Brian PH, Lukins BS (1990) Comparative dose-response relationships and acceptability of warfarin, brodifacoum and phosphorus to feral pigs. Aust. Wildl. Res. **17:** 101-112 (published)
Warfarin	Feral pigs (*Sus scrofa*)	Field trials (Australia)	0.09% Warfarin baits offered for a period of 57 days at 69 sites, controlled area: 94 km^2.	Only 2 (pregnant sows) of 189 pigs were believed to have survived the program which was equivalent to 98.9% reduction.	**A5.2/15:** Saunders G, Kay B, Parker B (1990) Evaluation of a warfarin poisoning program for feral pigs (*Sus scrofa*). Aust. Wildl. Res. **17:** 525-533 (published)

The following references identified in a literature search to contain information on Warfarin were considered to lack in relevance upon evaluation, and were thus omitted from this summary:

Author (year)	Title	EBRC ref. no.
Rehman AB et al. (1983)	Comparative haematological studies on *Tatera indica* with three anticoagulant compounds	192
Lund M (1981)	Comparative effect of the three rodenticides warfarin, difenacoum and brodifacoum on eight rodent species in short feeding periods	259
Arora KK et al. (1987):	Rodent control in commercial grain warehouses in India	115
Singh R et al. (1989):	The susceptibility of house rat to some anticoagulant rodenticides	116
Vissault J et al. (1976):	Sensitivity of three African murids to chlorophacinone and coumafene	159
Tanaka I et al. (1976):	Studies on the effectiveness of rodenticides. I. The comparative rodenticidal efficacy of several anticoagulants	166
Phillips D et al. (1976)	Warfarin: a question of balance	151
Greaves JH et al. (1974)	Properties of calciferol as a rodenticide	255
Rennison BD (1974)	Field trials of calciferol against warfarin resistant infestations of the Norway rat (*Rattus norvegicus*)	256
Piccinini RS et al. (1985):	Vampiricides for topical use on domestic animals and vampire bats	287
Moreira I et al. (1985):	Control of voles *Microtus lusitanicus* (Gerbe) and *M. duodecimcostatus* (Sel.-Long.) in orchards	303

Table A5-2: Summary table of experimental data and review articles on resistance against Warfarin in the target organisms (commensal and field rodents). Generally, the substance functions as a rodenticide (PT 14) and the envisaged field of use is pest control (MG03).

Reference (author, year)	Area or country	Species			Additional information
		Rattus norvegicus	*Rattus rattus*	*Mus musculus*	
A5.7/01: Pelz H-J, Hänisch D, Lauenstein G (1995) Resistance to anticoagulant rodenticides in Germany and future strategies to control *Rattus norvegicus*. Pestic. Sci. **43:** 61-67 (published)	Germany	x	-	-	resistance detected only in one isolated area in NW Germany; otherwise, in the whole of Germany rats are considered susceptible to Warfarin
A5.7/02: Boyle CM (1960) Case of apparent resistance of *Rattus norvegicus* Berkenhout to anticoagulant poisons. Nature **188**: p. 517 (published)	Scotland	x		-	first report of resistance in rats, found on one farm in Scotland.
A5.7/03: Myllymäki A (1995) Anticoagulant resistance in Europe: appraisal of data from 1992 EPPO questionnaire. Pestic. Sci. **43:** 69-72 (published)	Denmark	x	x	x	determination of Warfarin resistance was based on an EPPO questionnaire to its member countries. However, replies were inconsistent and lack a description of the extent and origin of the stated resistance.
	Finland	x	-	x	
	France	x	x	x	
	Germany	x	x	x	
	Great Britain	x	x	x	
	Netherlands	x	-	-	
	Italy	x	-	-	
A5.7/04: Jackson WB, Ashton AD (1995) Extended summary RRAC symposium anticoagulant resistance in North America. Pestic. Sci. **43:** 95-96 (published)	North America	most areas: < 20 % Chicago: 60 – 70 % of rats resistant		x	50 resistance areas, mostly urban.
A5.7/05: Misenheimer TM, Suttie JW (1990) Warfarin resistance in a Chicago strain of rats. Biochem. Pharm. **40:** 2079-2084 (published)	Chicago	x		-	investigation of one Warfarin-resistant rat strain trapped in Chicago.
A5.7./06: Greaves JH (1970) Warfarin-resistant rats in Britain. Agr. Sci. Rev. **8:** 35-38 (published)	Great Britain	x		-	resistance in three areas: Scotland (800 miles^2) Montgomeryshire/ Shropshire (1000 miles^2), Kent (40-50 miles^2)

The following references identified in a literature search to contain information on Warfarin were considered to lack in relevance upon evaluation, and were thus omitted from this summary:

Author (year)	**Title**	**EBRC ref. no.**
Cowan, D. et al. (1995)	The impact of resistance on the use of second-generation anticoagulants against rats on farms in Southern England	551
Bishop, J.A. et al. (1976)	The size and age structure of rural populations of *Rattus norvegicus* containing individuals resistant to the anticoagulant poison warfarin	296
Smith, R.H. et al. (1986)	Resistance to anticoagulant rodenticides the problem and its management	306
MacNicoll, A.D. (1993)	Anticoagulant rodenticides tolerance and resistance	307
Quy, R.J. et al. (1992)	Bait avoidance and effectiveness of anticoagulant rodenticides against warfarin- and difenacoum-resistant populations of Norway rats (*Rattus norvegicus*)	77
Greaves, J.H. et al. (1973)	Warfarin resistance and DT diaphorase activity in the rat	24
Hoque, M.M. (1983)	Development of an anticoagulant resistance monitoring program for Philippine rats	208
Sutcliffe, F.A. et al (1990)	Hepatic microsomal warfarin metabolism in warfarin-resistant and susceptible mouse straines: influence of pretreatment with cytochrome P-450 inducers	46
Bishop, J.H. et al. (1976)	The size and age structure of rural populations of *Rattus norvegicus* containing individuals resistant to the anticoagulant poison warfarin	296
Fasco, M.J. et al. (1983)	Formation of hydroxyvitamin K by vitamin K epoxide reductase of warfarin-resistant rats Vitamin K metabolites	1
Trivedi, L.S. (1988)	Normal and warfarin-resistant rat hepatocyte metabolism of vitamin K 2,3-epoxide: evidence for multiple pathways of hydroxyvitamin K formation	3
Zimmermann, A. et al. (1973)	Biochemical basis of hereditary resistance to warfarin in the rat	489