

ENTWICKLUNG EINES  
MASSENSPEKTROMETRIEBASIERTEN  
IMMUNOASSAYS ZUR  
AUTHENTIFIZIERUNG UND  
QUANTIFIZIERUNG ERLAUBTER  
INSEKTENPROTEINE IN LEBENSMITTELN

Bundesinstitut für Risikobewertung  
Tobias Meisinger  
23-Nov-2023



- WP 2.1 – Identification of species marker peptides in non-sequenced species
- WP 2.2 – MS Characterisation of pure allergens
- WP 2.3 – Method development of an immunoaffinity LC-MS/MS assay
- WP 2.4 – Method development of a lateral flow assay
- WP 2.5 – Testing of processed samples

Work packages	BfR	SIG	Nestlé	year 1												year 2												year 3											
	NMI	HSAS	Charité	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Coordination (BfR)				←-----→																																			
<b>2. Development and Validation MS and LFA</b>																																							
2.1 Bioinformatic sequence analysis (SIG)				←-----→																																			
2.2. MS Charakterisation of pure allergens from WP 1.2				█																																			
2.3 Method development MS (SIG)				█																																			
2.4 Method development LFA (SIG)				█																																			
2.5 Testing of processed samples/Matrix influence (SIG)				█																																			
2.5. Method transfer IPMS (SIG, Nestlé)				█																																			

## GENERAL APPROACH

Heterogeneous  
Phase Digestion



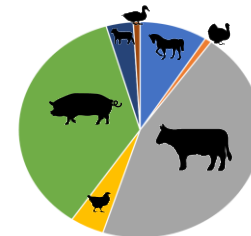
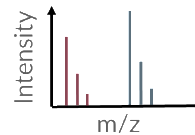
Cross-Species  
Immunoenrichment



GSGGTAEHPFTVEEFVLPK  
 ESGGTAEHHTVEEFVLPK  
 VVQQESGETAEHPFTVEEFVLPK  
 AEHPFIVEEFVLPK  
 TIHHPFSVEEYVLPK  
 TIQHPFTVEEYVLPK  
 TIQHPFSVEEYVLPK  
 IQHSFSVEEYVLPK



LC-MS/MS  
Identification & Quantification









well elucidated  
species....



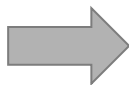
# SIGNATOPE

## TARGET SPECIES PROTEOMES ARE INCOMPLETE

Species	UniProtKB # entries (Nov-2021)	% proteome estimate*	Genome sequenced?	Genome quality**
<b><i>Tenebrio molitor</i></b> TENMO Yellow mealworm 	634	2.8%	Yes	Scaffold
<b><i>Locusta migratoria</i></b> LOCM1 Locust 	1,559	7.1%	Yes	Contig
<b><i>Gryllodes sigillatus</i></b> GRYS1 Cricket 	37	0.2%	No	n/a Scaffold
<b><i>Alphitobius diaperinus</i></b> ALPDA Mealworm 	47	0.2%	No	n/a
<b><i>Acheta domesticus</i></b> ACHDO House cricket 	159	0.7%	Yes (no publication?)	Contig
<b><i>Hermetia illucens</i></b> HERIL Black soldierfly 	17,599	100%	Yes	Annotated genome

\*estimates by size of *Drosophila melanogaster* proteome

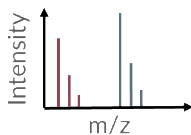
\*\*Single reads < Contig < Scaffold < Chromosome < Annotated



Sequence homology approach required!

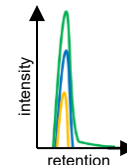
## SIMPLIFIED WORKFLOW FOR POTENTIAL TARGET CONFIRMATION

---



GSGGTAEHPFTVEEFVLPK  
ESGGTAHHFTVEEFVLPK  
VVQQESGETAEHPFTVEEFVLPK

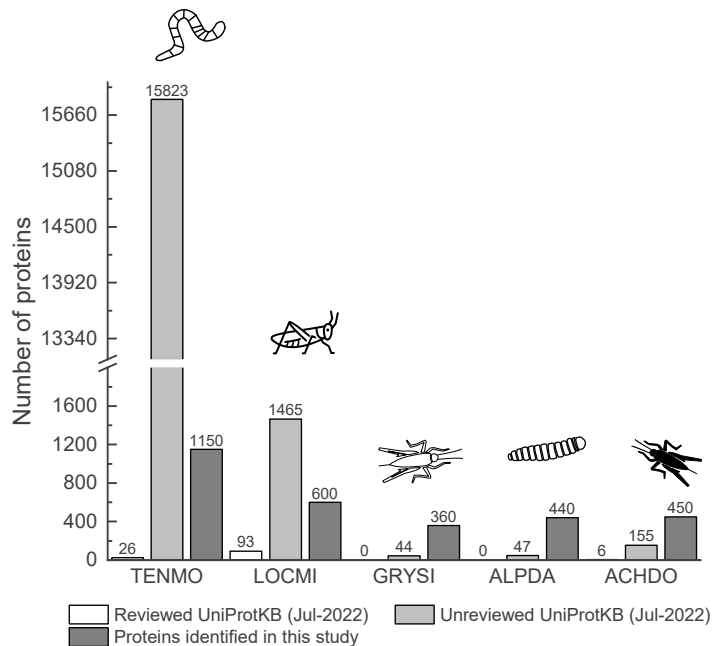
NP\_001103265.1|alpha-2-macroglobulin precursor [Bos taurus]  
GSGGTAEHPFTVEEFVLPK



**Unique:**  
Exists in only **one target species**  
Not existent in livestock species  
Not existent in other arthropods besides insects

**Might exist** in a small number of other insects

## EXPANSION OF KNOWN PROTEOMIC DATA > POTENTIAL SPECIES MARKER



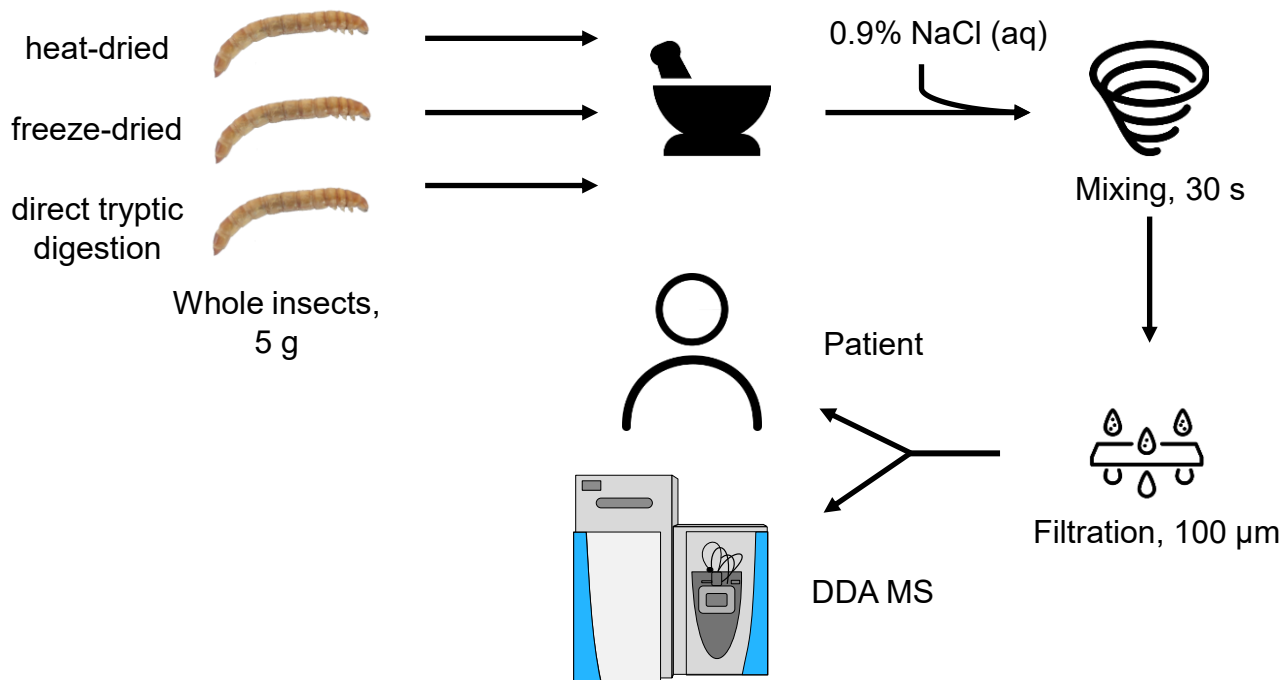
Species	Protein name
<i>Tenebrio molitor</i>	Larval cuticle protein F1
<i>Locusta migratoria</i>	Vitellogenin A
<i>Grylodes sigillatus</i>	Arginine kinase, Spermatophylax protein C
<i>Alphitobius diaperinus</i>	Larval cuticle protein A3A, Hemocyanin C
<i>Hermetia illucens</i>	Cuticle protein, Superoxide dismutase
<b>Insecta</b>	Tropomyosin



- WP 2.1 – Identification of species marker peptides in non-sequenced species
- WP 2.2 – MS Characterisation of pure allergens
- WP 2.3 – Method development of an immunoaffinity LC-MS/MS assay
- WP 2.4 – Method development of a lateral flow assay
- WP 2.5 – Testing of processed samples

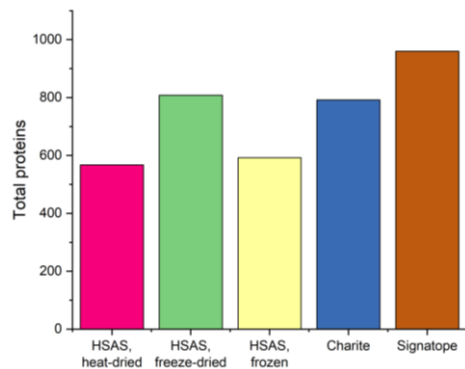
Work packages	BfR	SIG	Nestlé	year 1												year 2												year 3											
	NMI	HSAS	Charité	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Coordination (BfR)				←-----→																																			
<b>2. Development and Validation MS and LFA</b>																																							
2.1 Bioinformatic sequence analysis (SIG)	-----→																																						
2.2 MS Charakterisation of pure allergens from WP 1.2	-----→																																						
2.3 Method development MS (SIG)	-----→																																						
2.4 Method development LFA (SIG)	-----→																																						
2.5 Testing of processed samples/Matrix influence (SIG)	-----→																																						
2.5. Method transfer IPMS (SIG, Nestlé)	-----→																																						

## WHAT ABOUT ALLERGENICITY? - PRICK SOLUTIONS

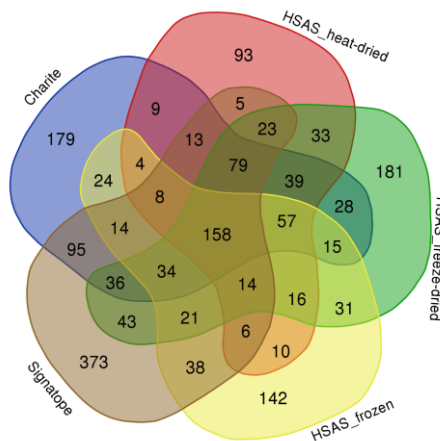




## HOMOLOGUE ALLERGENS WERE IDENTIFIED



Similar IDs of proteins



Strong overlap in all samples, Direct tryptic digestion leads to more/different IDs

### Identified homologue allergens

Alpha-amylase	Heat shock proteins
---------------	---------------------

<b>Arginine kinase</b>	Paramyosin
------------------------	------------

Chitinase	Profilin
-----------	----------

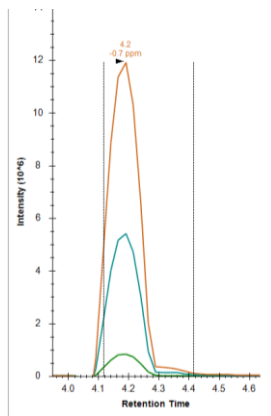
Cytochrome C	Thioredoxin
--------------	-------------

Ferritin	<b>Tropomyosin</b>
----------	--------------------

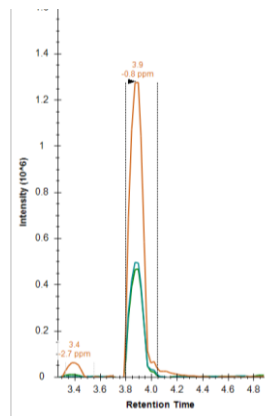
Glutathione S-transferase	Troponin
---------------------------	----------



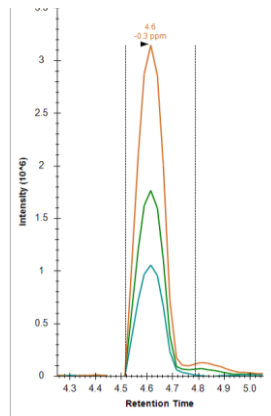
## INSECT MARKER DISPLAY SHARP PEAKS IN ENDOGENOUS SAMPLES



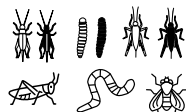
Arginine kinase  
*G. sigillatus*  
Also found in one ant



Spermatophylax  
protein C  
*G. sigillatus*  
unique



Tropomyosin  
*Insecta*



- PRM analyses of insect digests
- Confirm *in silico* and in-house proteomic results



Generation of standard peptides for quantification and antibodies

## MS OPTIMIZATION LEAD TO SPECIFIC AND STRONG SIGNALS

- Check antibody specificity

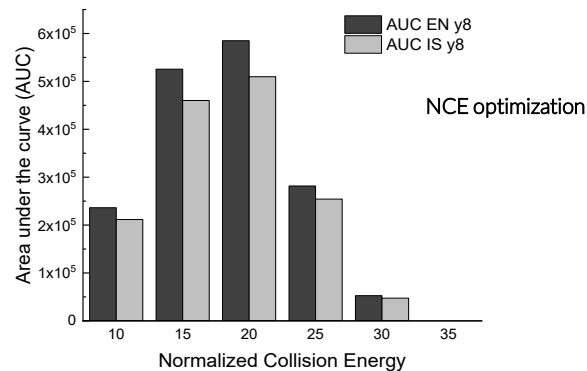
➔ No cross-reactivities observed

- Collision energy optimization

➔ NCEs with highest peak intensities could be determined

Antibody specificity in SIS mix

	ALPDA	TENMO	HERIL	LOCFI	GRYSI	GRYSI	INSECTS
ALPDA ab406 animal 1	196374	2219	0	n/a	0	0	0
TENMO ab407 animal 1	0	696224	0	n/a	n/a	n/a	0
HERIL ab408 animal 2	n/a	n/a	3943496	0	n/a	n/a	0
LOCFI ab410 animal 1	n/a	n/a	n/a	1919176	0	n/a	0
GRYSI ab411 animal 1	n/a	n/a	n/a	0	3432073	n/a	3282
GRYSI ab412 animal 1	0	n/a	0	0	n/a	1915429	2577
INSECTS ab413 animal 1	n/a	2138	n/a	n/a	2320	n/a	1572453
blank	n/a	n/a	2594	0	2762	0	0

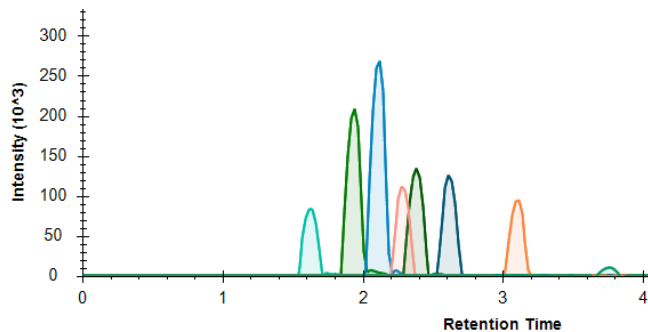
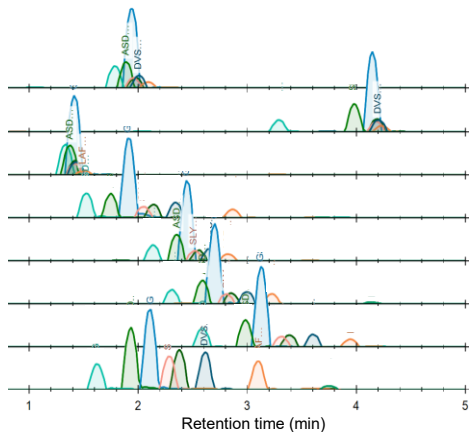




# SIGNATOPE

## GRADIENT OPTIMIZATION LED TO FAST SAMPLE ANALYSIS

---



Optimal separation and run time identified from 10 different gradients

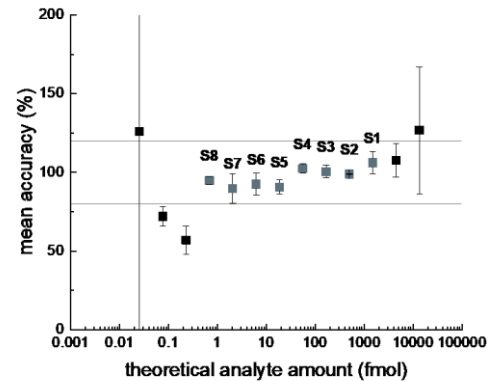
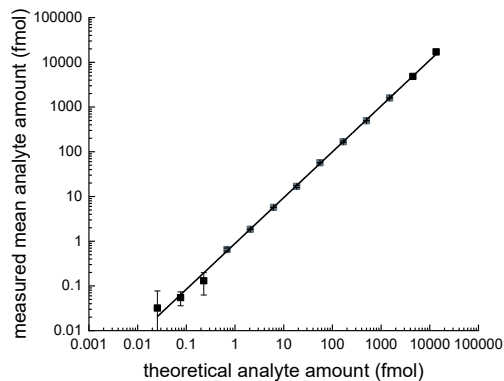
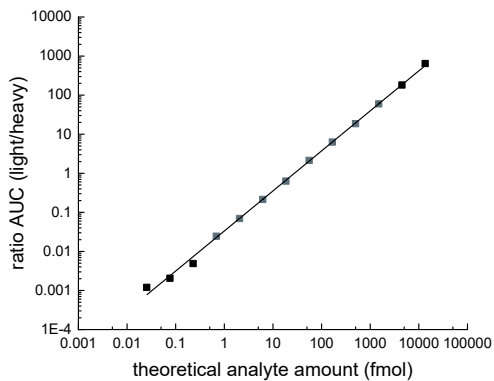


Cycle time of 6 min



# SIGNATOPE

## CALIBRATOR OPTIMIZATION LED TO ROBUST STANDARD CURVES



Quantification possible through eight calibrator levels



S1-S8, range 0.69 fmol – 1500 fmol, 80-120 % accuracy



# SIGNATOPE

## METHOD DEVELOPMENT FAILED

---







- LFA development tested with *Insecta* species marker

➔ Antibodies do not recognize peptide in sandwich-complex










- WP 2.1 – Identification of species marker peptides in non-sequenced species
- WP 2.2 – MS Characterisation of pure allergens
- WP 2.3 – Method development of an immunoaffinity LC-MS/MS assay
- WP 2.4 – Method development of a lateral flow assay
- WP 2.5 – Testing of processed samples

Work packages	BfR	SIG	Nestlé	year 1												year 2												year 3											
	NMI	HSAS	Charité	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Coordination (BfR)				←-----→																																			
<b>2. Development and Validation MS and LFA</b>				-----																																			
2.1 Bioinformatic sequence analysis (SIG)				-----																																			
2.2. MS Charakterisation of pure allergens from WP 1.2				-----																																			
2.3 Method development MS (SIG)				-----																																			
2.4 Method development LFA (SIG)				-----																																			
2.5 Testing of processed samples/Matrix influence (SIG)				-----																																			
2.5. Method transfer IPMS (SIG, Nestlé)				-----																																			

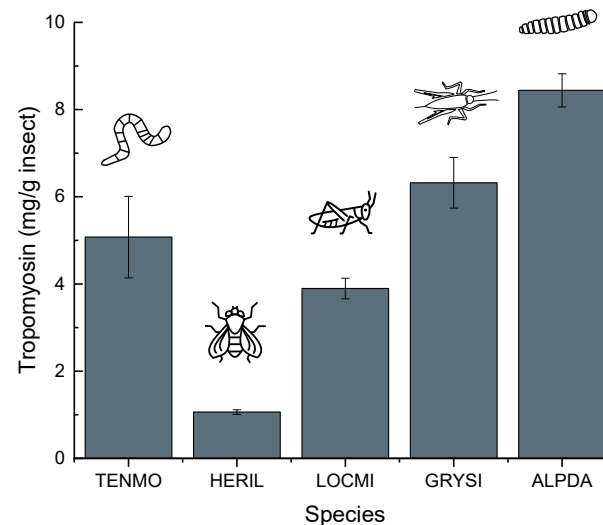





## INSECT SPECIES MARKER QUANTIFIABLE IN INSECT SAMPLES

Insect species marker








	Species	Identifier	Mean amount (mg/g insect) (n=3)	SD (n=3)
	GRYSI	AK	0.28	0.06
	GRYSI	SP1C	0.50	0.02
	HERIL	HICP	0.04	0.00
	ALPDA	HCC	4.59	0.32
	ALPDA	LCPA3A	6.27	0.61
	TENMO	LCPF1	0.005	0.001
	LOCFI	VTGA	22.02	2.93

Generic insect marker



➔ Tropomyosin is similar abundant in all species except *Hermetia illucens*

## INSECT SPECIES MARKER OBSERVABLE IN PROCESSED FOOD SAMPLES

Species	Identifier	Mean amount (µg/mg insect) (n=3)	Observable in which cookie sample?	Mean amount protein (ppm)	Expected amount (ppm)
 GRYSI	AK	0.28	100 ppm	0.010	0.008
 GRYSI	SP1C	0.50	20 ppm	0.001	0.012
 HERIL	HICP	0.04	20 ppm	0.002	0.0009
 ALPDA	HCC	4.59	100 ppm	0.062	0.11
 ALPDA	LCPA3A	6.27	100 ppm	0.005	0.17
 TENMO	LCPF1	0.005	-	-	0.0001
 LOCMI	VTGA	22.02	-	-	0.48
INSECTA	TPM	4.96*	600 ppm	0.292	0.605

\* Mean of five insect species

Processed cookie samples



0 ppm



30 ppm



120 ppm



600 ppm



Some insect marker were observed in 120 ppm but not in 600 ppm cookies



Improvement of sample homogeneity required

- WP 2.1 – Identification of species marker peptides in non-sequenced species
- WP 2.2 – MS Characterisation of pure allergens
- WP 2.3 – Method development of a immunoaffinity LC-MS/MS assay
- WP 2.4 – Method development of a lateral flow assay
- WP 2.5 – Testing of processes samples

Work packages	BfR	SIG	Nestlé	year 1							year 2							year 3																													
	NMI	HSAS	Charité	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36								
Coordination (BfR)	←																																														
<b>2. Development and Validation MS and LFA</b>																																															
2.1 Bioinformatic sequence analysis (SIG)																																															
2.2. MS Charakterisation of pure allergens from WP 1.2																																															
2.3 Method development MS (SIG)																																															
2.4 Method development LFA (SIG)																																															
2.5 Testing of processed samples/Matrix influence (SIG)																																															
2.5. Method transfer IPMS (SIG, Nestlé)																																															



# SIGNATOPE

## OUTLOOK

---

- Test assay in other food samples
- Further assay improvements
- Recovery tests in different food matrices for tropomyosin
- Test *in vitro* digested food samples for allergenicity
- Assay transfer to Nestlé

THANK YOU!



**Oliver Pötz**  
**Hannes Planatscher**  
Amelie Vogt  
Andreas Steinhilber  
Anja Tausch  
Cornelia Sommersdorf  
Hanna Hitzelberger  
Helen Hammer  
Isabel Bek  
Katharina Bendel  
Lena Heumesser  
Robin Kretz  
Salina Amos  
Wael Naboulsi



**Ulrich Rothbauer**



**Dieter Stoll**  
**Eva-Marie Ladenburger**



**Albert Braeuning**  
Cristiano Garino  
Hermann Broll



Kirsten Beyer  
Lara Meixner  
Meilan Zuo



**Markus Templin**  
**Thomas Joos**



Bundesanstalt für  
Landwirtschaft und Ernährung



Bundesministerium  
für Ernährung  
und Landwirtschaft

ALLERGEN-PRO  
FKZ 281A304B18