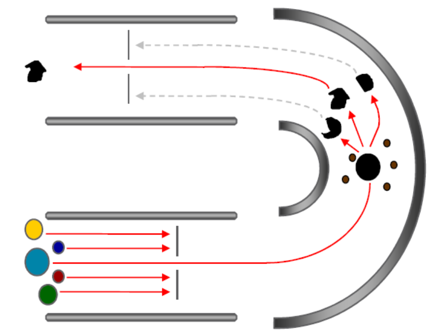




Various Analytical Approaches for Tracking and Tracing in the Meat Area

F. Schwägele, W. Jira, S. Münch, K.-H. Schwind

Max Rubner-Institut (MRI), Federal Research Institute of Nutrition and Food,
Department of Safety and Quality of Meat, Location Kulmbach, Germany

With **increasing global distribution of feed, food and ingredients** the different countries in our world have never been before more dependent on each other with respect to their food supply. *(Wall, 2009)*

Consequently

An united approach with consistent standards based on sound science and robust controls is necessary to ensure consumers‘ health and to maintain consumers‘ confidence.

The General European Food Law

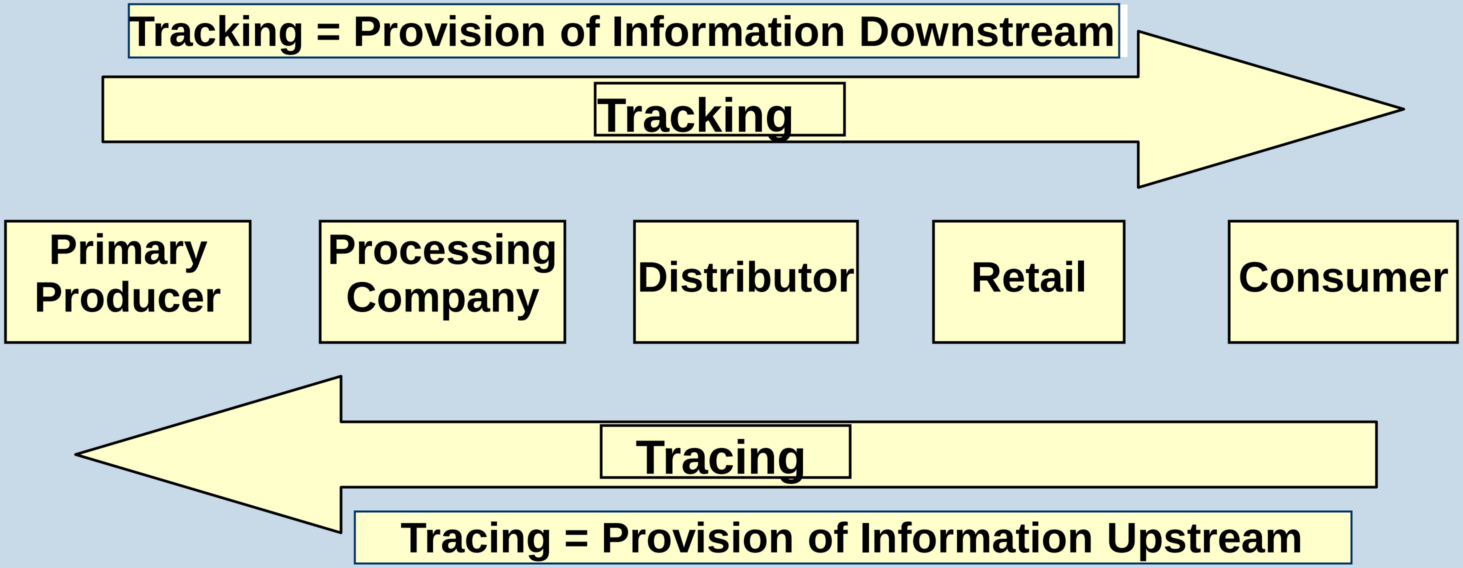
- **Regulation (EC) 178 (2002)** of the European Parliament and the Council published on 28 January 2002:
- Outlines the general principles and requirements of food law
- Establishes the European Food Safety Authority (EFSA)
- Provides procedures in matter of food safety, i.e. among other things the implementation of **traceability systems** in the food and feed supply chains in Europe
- **Article 18** of the regulation referring to **traceability of food and feed** is valid since **1 January 2005**

Indispensable requirements for every food business

- Appropriate process control
- Biosecurity
- Adequate traceability
- Good hygiene and manufacturing practices

(Andrée et al., 2010)

Food safety and **quality** is assigned to **various analytical approaches** along the whole food chain downstream (tracking) from primary production to the consumer and upstream (tracing) from the consumer to the primary production *(Schwägele, 2005).*



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Various analytical approaches for tracking and tracing in the meat area

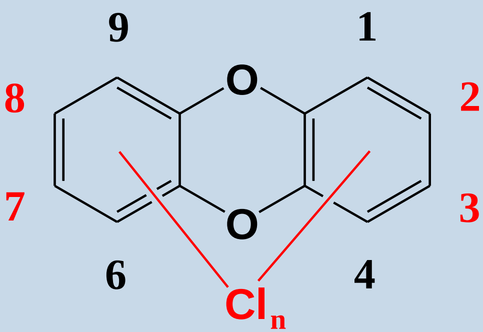
Analysis of

- Organic residues and contaminants
- Heat-induced contaminants
- Animal species quantitation by real-time PCR
- Allergen analysis by HPLC-MS/MS

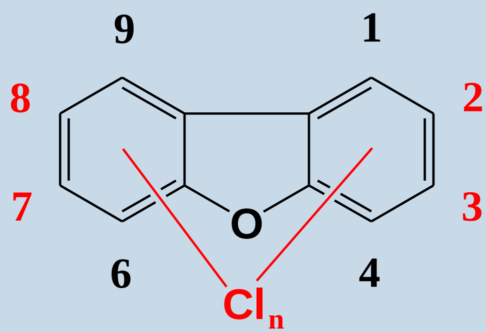
Organic residues and contaminants

(Andrée et al., 2010)

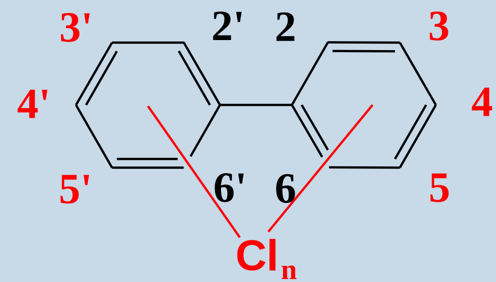
PCDDs, PCDFs and PCBs : Toxic compounds



PCDD
Poly**c**hlorinated **D**ibenzo-p-**d**ioxins



PCDF
Poly**c**hlorinated **D**ibenzo**f**urans

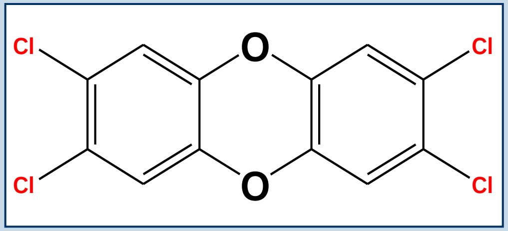


PCB
Poly**c**hlorinated **B**iphenyls

12 dioxin-like PCBs

Important for analysis: **29** congeners **with toxic behaviour** (of in total 75+135+209=419)

Classification of 2,3,7,8-TCDD by IARC (Int. Agency for Research on Cancer) Group 1: carcinogenic to humans



The status survey study: Monitoring of German feed and food by means of representative samples

Toxicity Equivalence (TEQ) concept; Toxic Equivalent Factor (TEF)

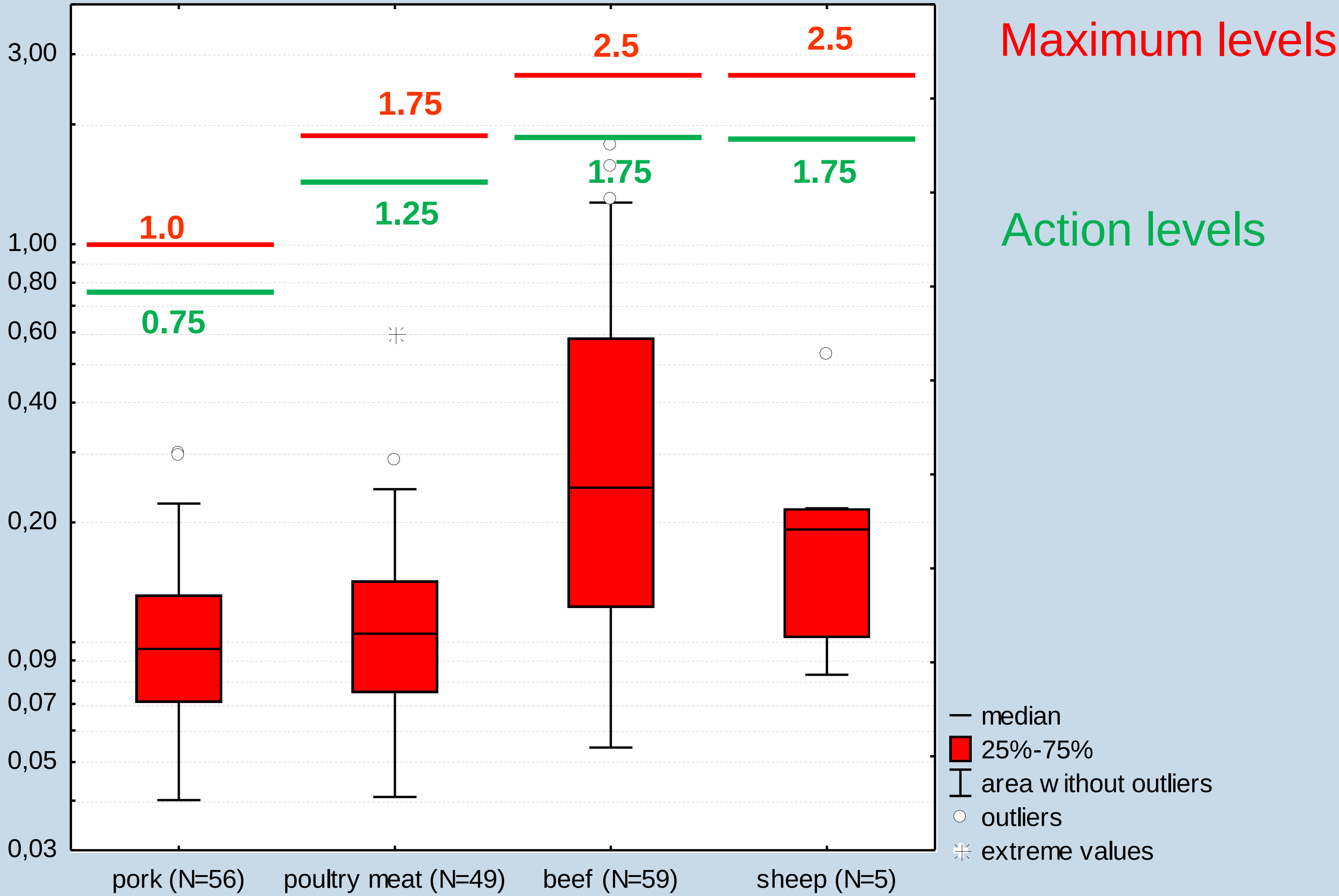
- Project of the Federal Ministry of Food, Agriculture and Consumer Protection in Germany (4 years)
- Determination of the concentrations of **dioxins**, **dioxin-like PCBs** and **6 marker-PCBs** in the same sample
- Investigation of highly representative samples (at least 200 samples of each matrix) by specific extraction and **GC-MS**

MRI Kulmbach (coordination)
feed, meat/meat products, eggs

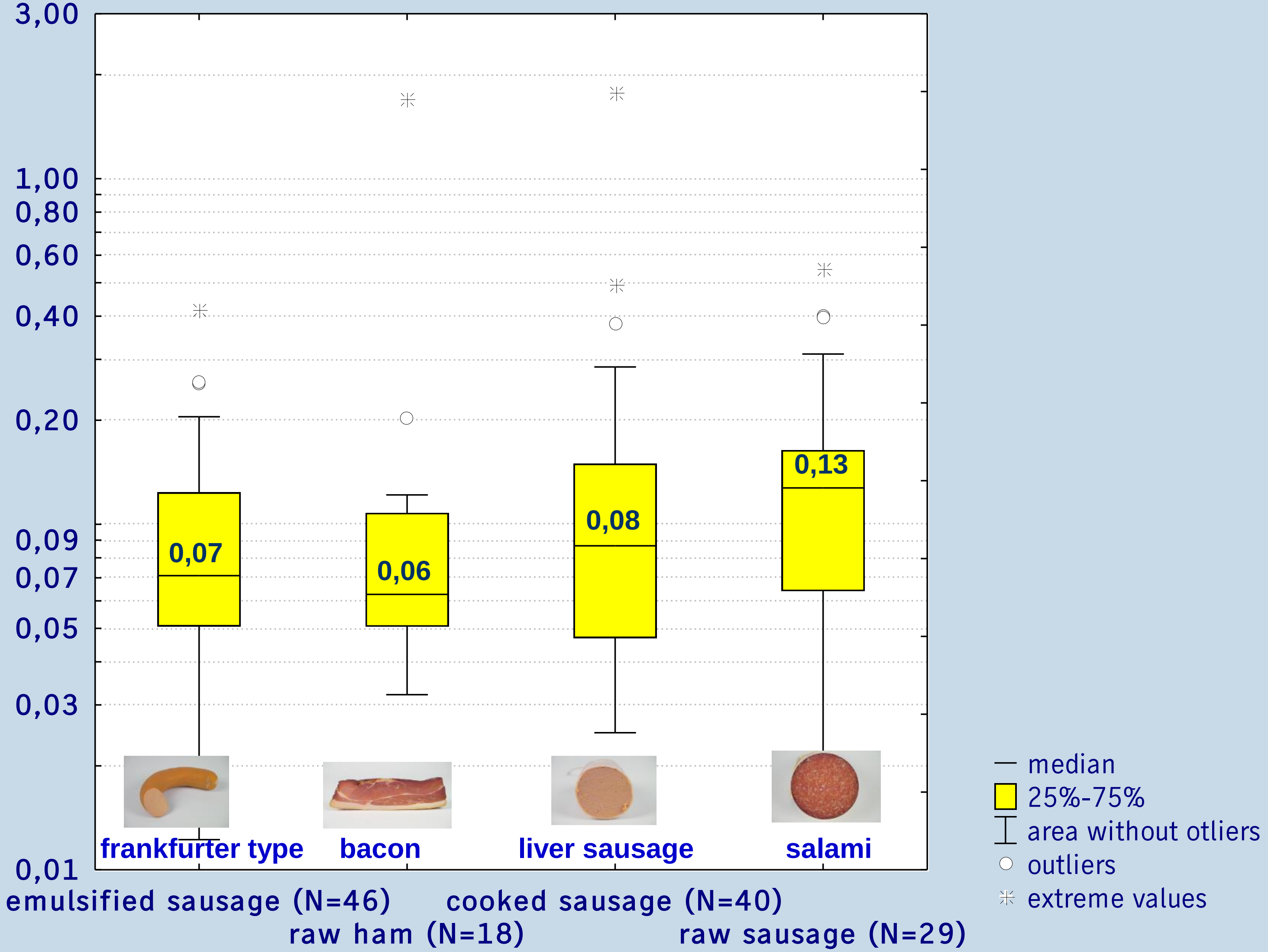
↕

MRI Kiel/Hamburg
milk/milk products, fish

WHO-PCDD/F-TEQ [ng/kg fat] in different types of meat in Germany



WHO-PCB-TEQ [ng/kg fat] in different meat products in Germany



Conclusion: Intake of PCDD/Fs and dl-PCBs from meat and meat products

- About 70-80% of human dioxin and dioxin-like PCB intake originates from food of animal origin
- Tolerable weekly intake (TWI) of dioxins and dioxin-like PCB:

  Scientific Committee on Food (SCF):
  14 pg WHO-TEQ/kg body weight per week
  that means 2 pg WHO-TEQ/kg body weight per day

- **Daily intake of the German consumer (70 kg body weight):**
  4 pg WHO-PCDD/F-PCB-TEQ
  That means an exhaustion of ≈ 3% of the listed TWI


frankfuter type sausage


Raw ham (bacon)


Cooked liver sausage


Raw sausage (Salami)


pork


poultry


beef

Heat-induced contaminants

(Pöhlmann et al., 2012)

Smoke in meat products

- Smoking is one of the oldest technologies for the conservation of meat products
- Process of penetration of meat products by volatiles resulting from thermal destruction of wood *(Toth, 1982)*
- In Germany about 60% of meat products are smoked *(Frede, 2006)*

About **1100 different compounds** (analysis by GC-MS and LC-MS)

Positive smoke ingredients: aldehydes, carboxylic acids, phenolic substances (odour, flavour, antioxidative properties)

Negative smoke ingredients: Polycyclic Aromatic Hydrocarbons (PAH)

Polycyclic Aromatic Hydrocarbons

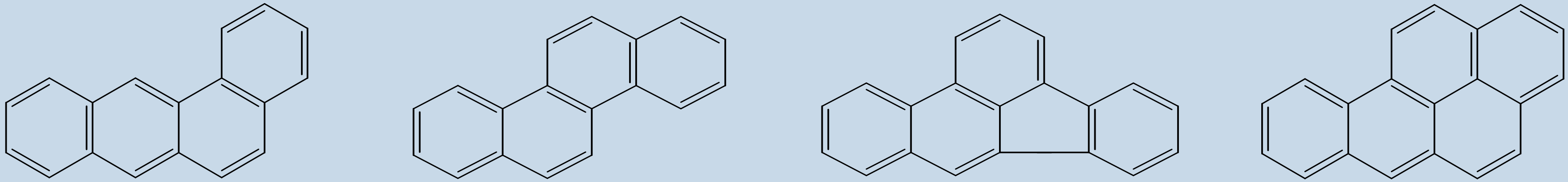
EU regulation 1881/2006:

⇨ 2 maximum levels in smoked meat products: PAH show carcinogenic properties

BaP: **5 µg/kg** until 31.08.2014
2 µg/kg as from 01.09.2014

PAH4: **30 µg/kg** until 31.08.2014
12 µg/kg as from 01.09.2014

= sum content of:



benzo[a]anthracene — chrysene — benzo[b]fluoranthene — benzo[a]pyrene

Factors influencing PAH contents in smoked meat products

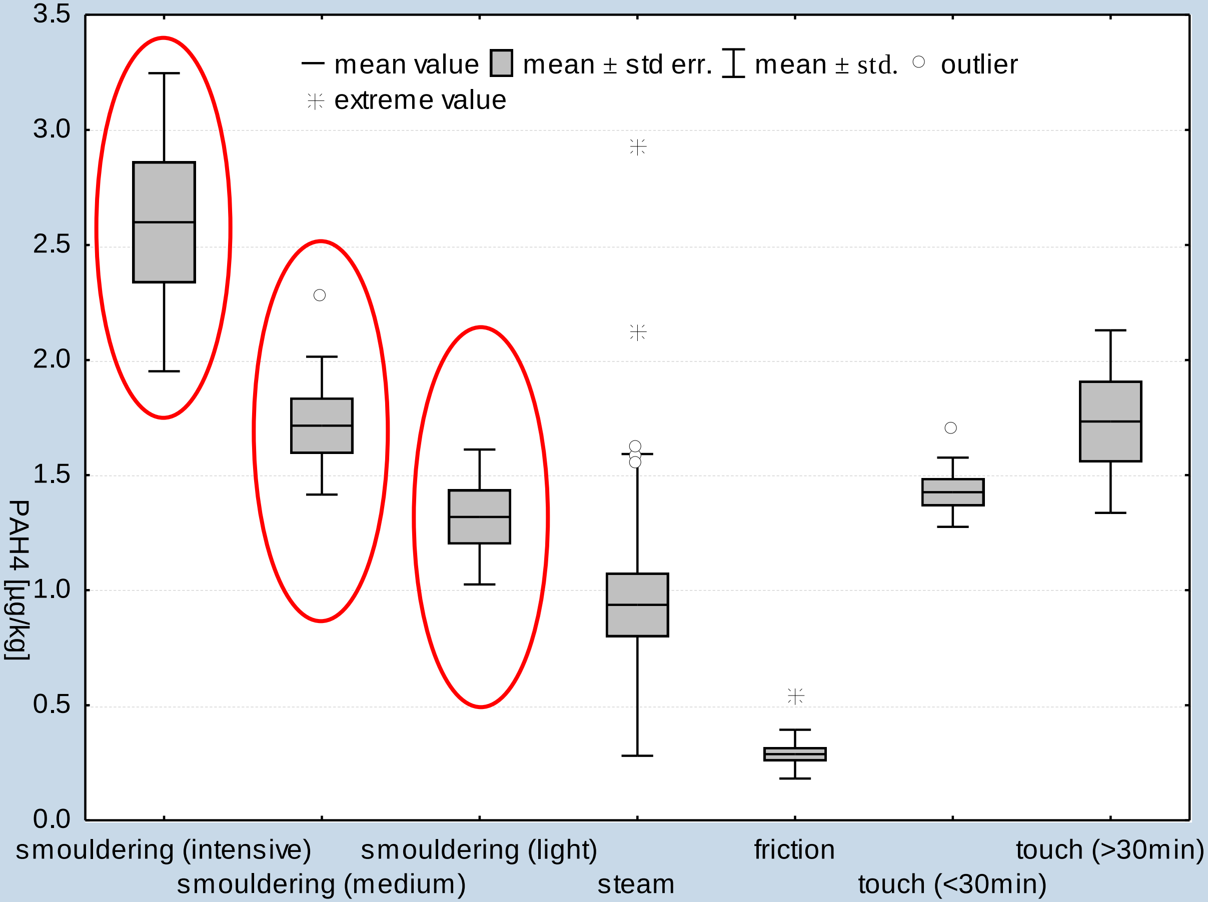
- Smoke generation method and its processing parameters:
  glow smoke, friction smoke, steam smoke, touch smoke

- Type of casing

- Fat content

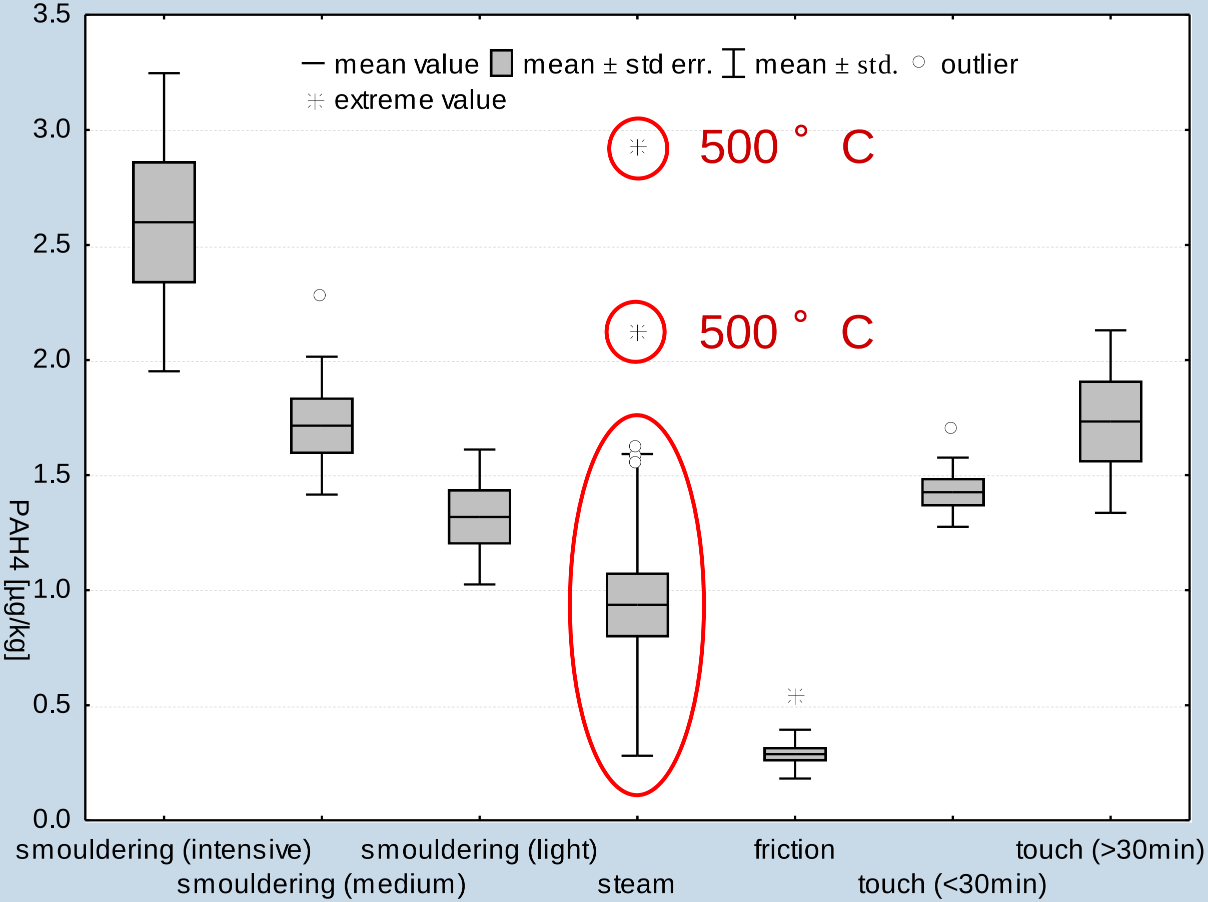
- Type of wood

PAH contents in Frankfurters applying different smoke generators



- Smouldering smoke: intensive > medium > light
- Steam smoke: PAH contents lower than for smouldering smoke
- Friction smoke: lowest PAH contents
- Touch smoke: PAH contents comparable to light and medium smoke

PAH contents in Frankfurters applying different smoke generators



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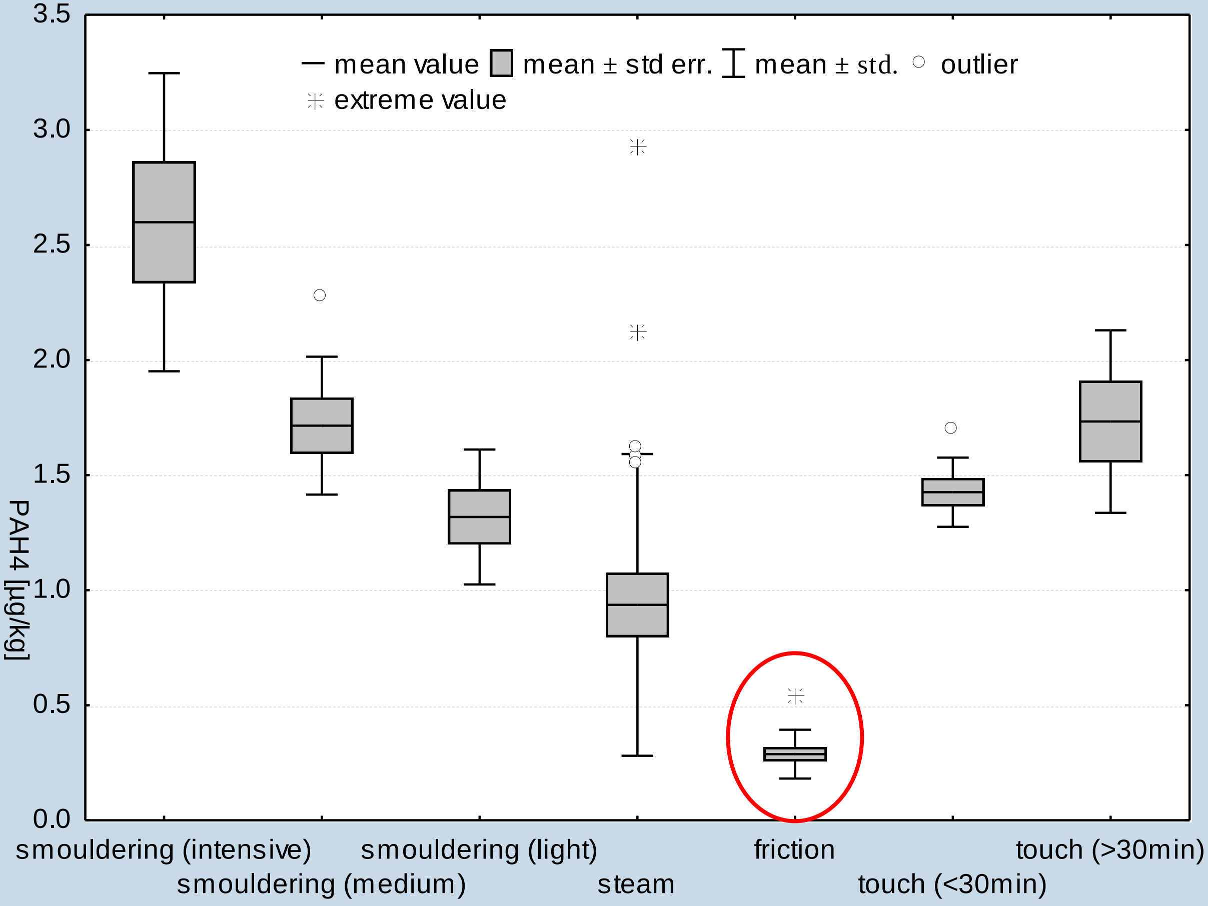

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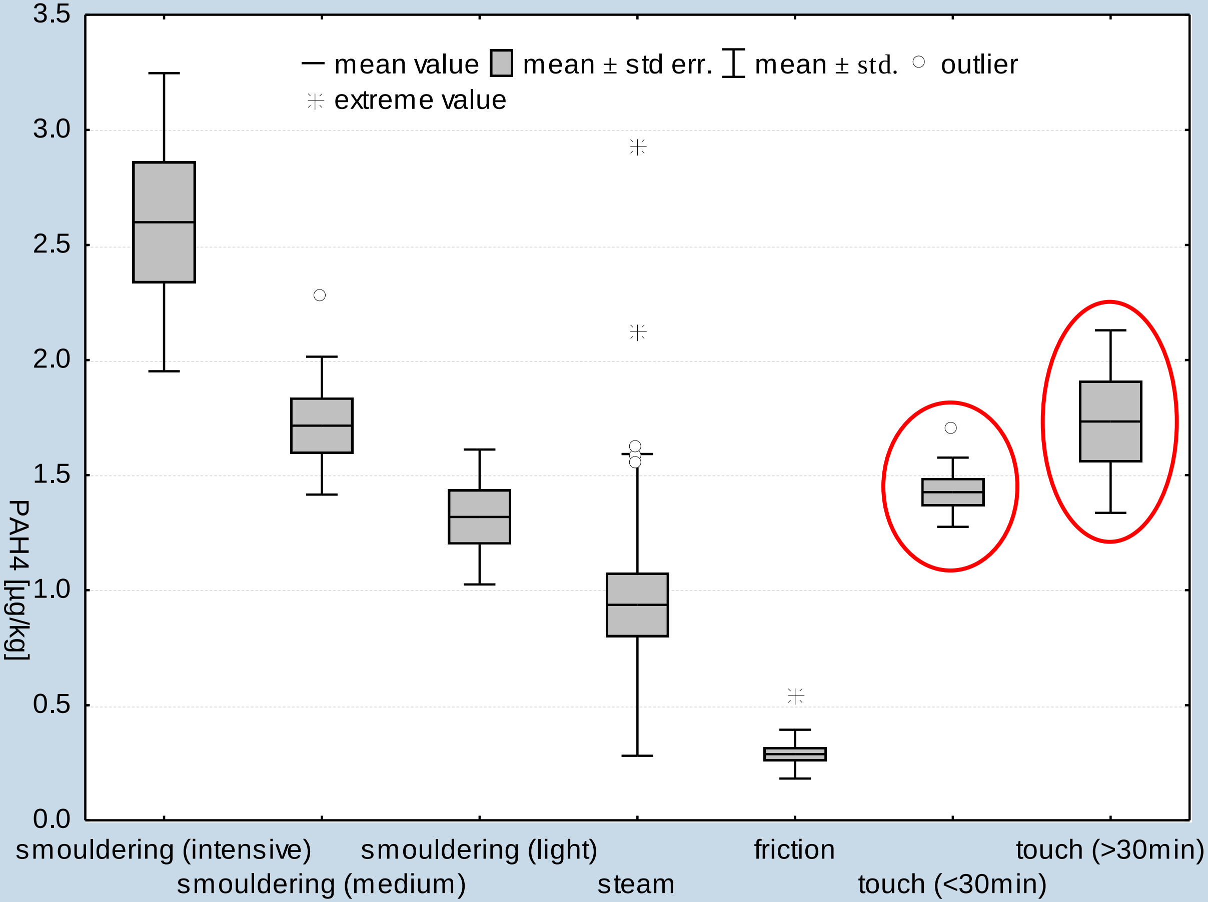

- Smouldering smoke:
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  PAH contents lower than for smouldering smoke
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  lowest PAH contents
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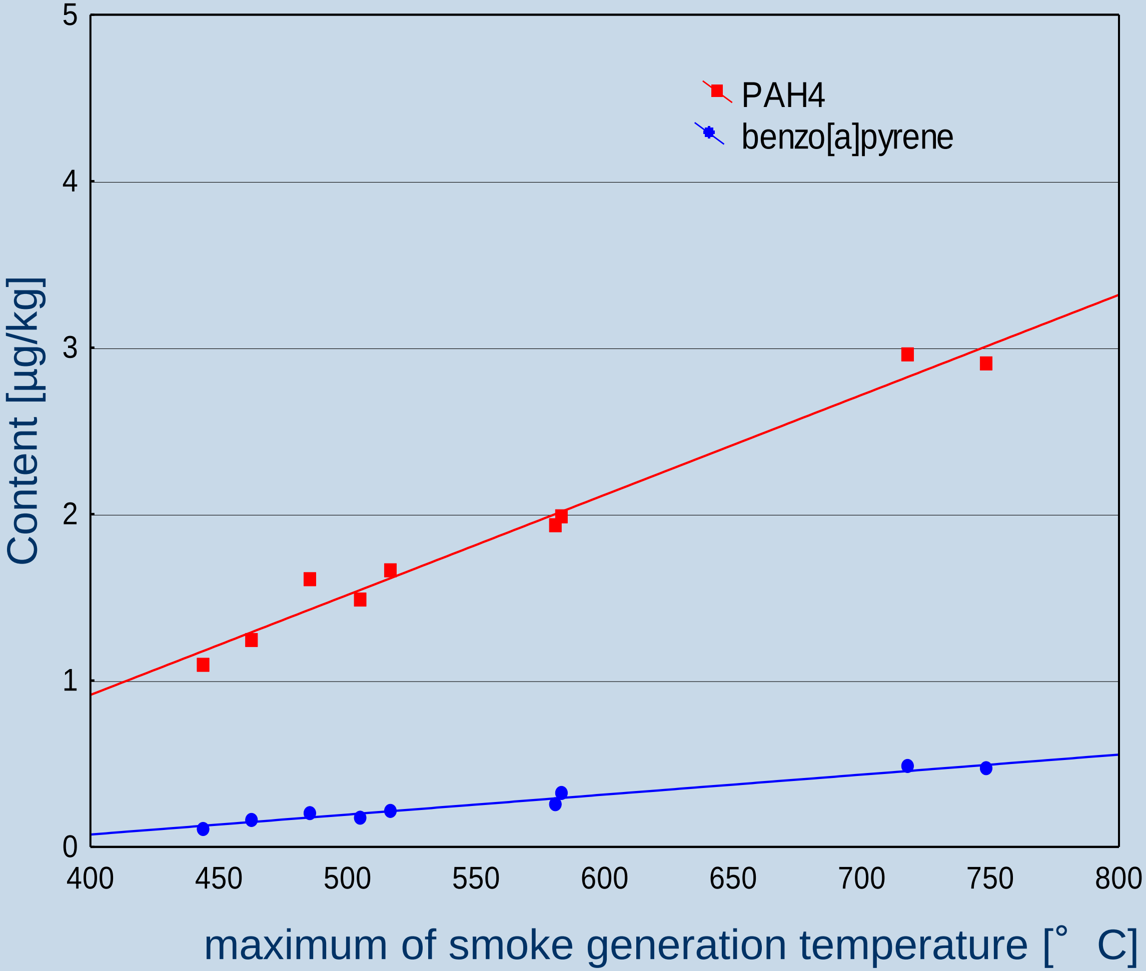
  PAH contents comparable to light and medium smoke

Correlation between maximum of smoke generation temperature [° C] and contents of „PAH4“ and benzo[a]pyrene [µg/kg] using glow smoke



- Increase in smoke generation temperature

⇓

Higher contents of „PAH4“ and benzo[a]pyrene

- „PAH4“:
  450 ° C: 1 µg/kg
  750 ° C: 3 µg/kg

⇓

Factor 3

Conclusion

- The use of modern smoke generators leads to low PAH contents far below the maximum levels
- The lowest PAH contents are observed for sausages smoked by means of friction smoke
- Lowering the contents of PAH compounds does not necessarily lead to a decrease in the amounts of phenolic substances
- The most important parameter influencing the PAH content using glow smoke is the smoke generation temperature

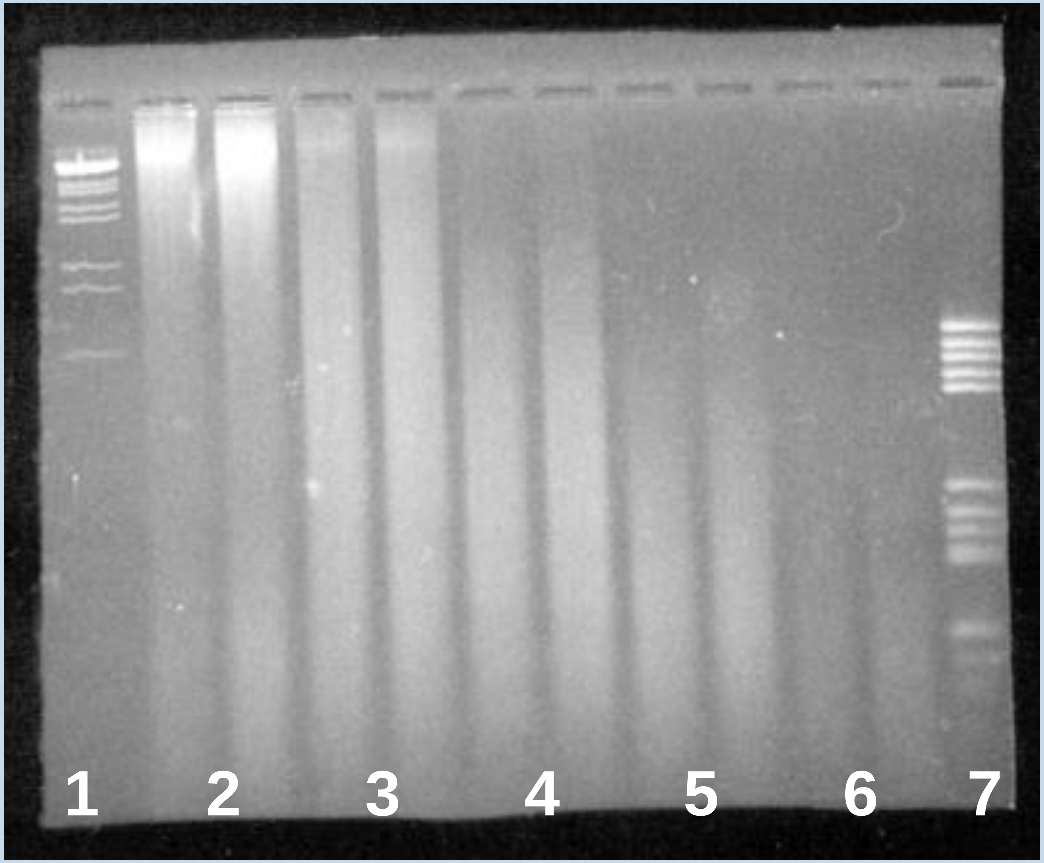
Animal species quantitation by real-time PCR

(Binke et al., 2005)

Real-time PCR

- Real-time PCR is a molecular biological technique used to amplify and simultaneously detect or quantify a target DNA-molecule
- A specific thermocycler is used, which is able to record the fluorescence-intensity caused by various fluorescent dyes in parallel to the DNA-amplification
- By use of suitable multiplex-PCR systems it is possible to identify and quantify up to 7 different species in a single assay (number of lasers and channels)

DNA fragmentation upon heat intensity



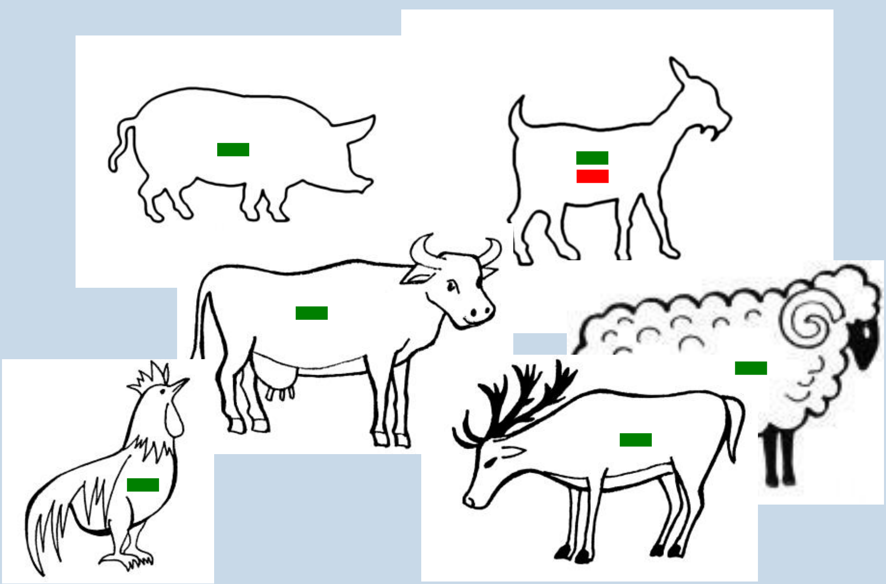
1. Marker 20 kbp – 500 bp
2. Home canned can
3. ¾ normal can
4. Normal can
5. Can for use under tropical conditions
6. Extremely heated product, Fc = 30
7. Marker 500 bp – 10 bp

Conclusion: Fragmentation of DNA is increasing according to heat intensity

Relative quantitation of goat

- Principle:

$$\text{Ratio} = \frac{CN_A \text{ (Target gene fragment = } \textit{beta-casein}\text{)}}{CN_A \text{ (Reference gene fragment = m}\textit{yostatin}\text{)}} >> \frac{\text{Goat meat percentage}}{\text{Total meat percentage}}$$



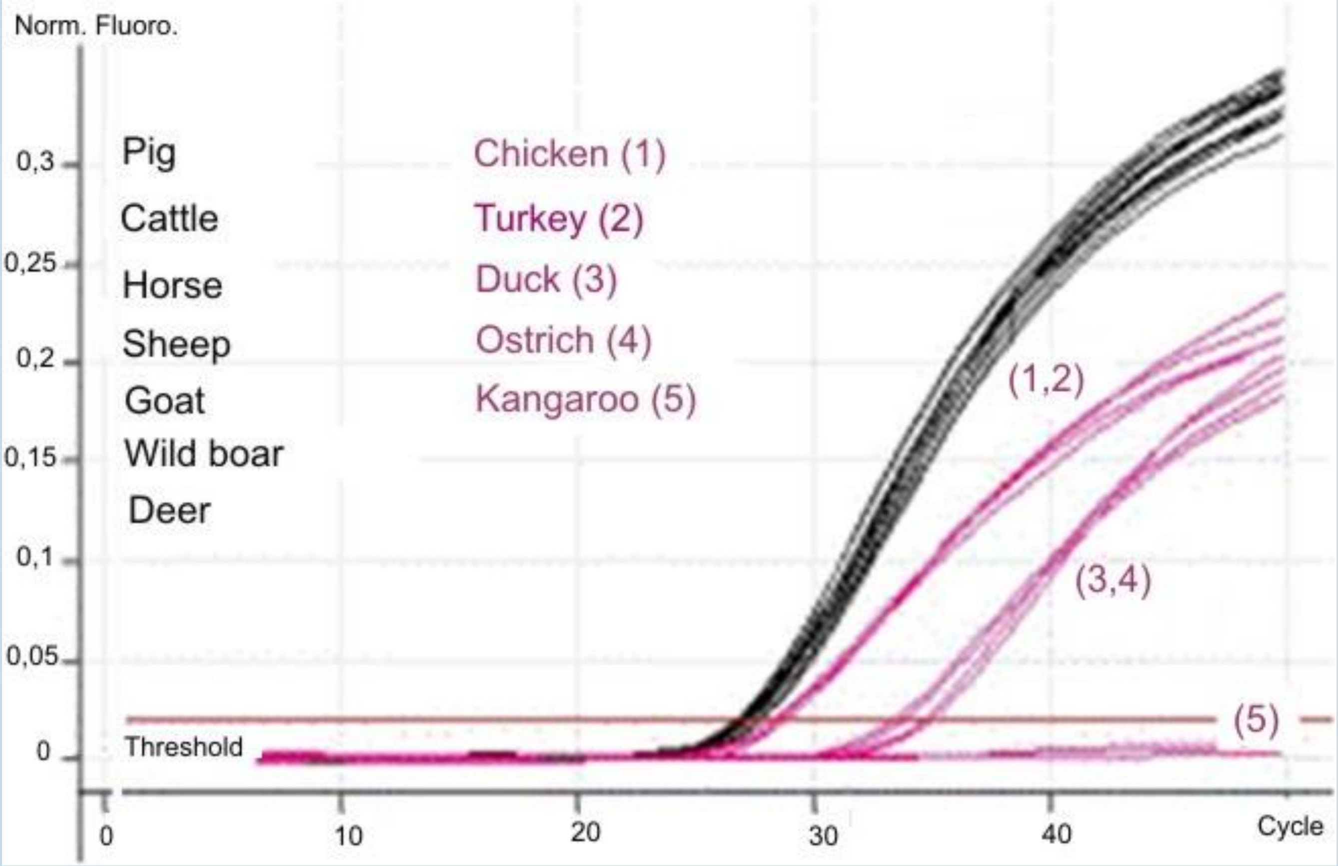
- *Beta-casein* gene sequence
- *Myostatin* gene sequence (= Inhibition control)

Myostatin gene sequences (*LAUBE et al*., 2002)

Quantitative PCR system for goat

Myostatin gene sequence (154 bp) as reference gene

Amplification course of the myostatin gene fragment (154 bp) for 12 animal species



Quantitative PCR system for goat

MRI

Validation of the test system: *beta-casein* (161 bp) ***/*** *myostatin (154 bp)*

	Quota [% Goat]	Actual [%] unheated	Actual [%] HCC	Actual [%] NC	Actual [%] TC
Emulsified Sausage 1	100*	109 ± 9	93 ± 11	114 ± 16	122 ± 22
Emulsified Sausage 2	100	77 ± 23	90 ± 16	82 ± 8	79 ± 18
Emulsified Sausage 3	50	57 ± 4	59 ± 17	56 ± 11	47 ± 8
Emulsified Sausage 4	20	13 ± 1	20 ± 6	19 ± 1	21 ± 7
Emulsified Sausage 5	2	1.9 ± 0.4	2.0 ± 0.7	2.0 ± 0.8	2.7 ± 1.2

* = without pig fat (25 % plant oil)

Conclusion: Relative quantitation based on the myostatin system is possible

Allergen analysis by HPLC-MS/MS

(Hoffmann et al., 2017)

Lupine and soy as allergens

- Already very small amounts (low ppm range) of lupine and soya protein can be dangerous to allergic persons (pea protein as an allergen is less relevant)

- The reference dose rates of the VITAL (*Voluntary Incidental Trace Allergen Labeling*) Expert Panel *(Taylor et al., Food and Chemical Toxicology, 63, 9-17 (2014))*: lupine: 4 mg protein; soy: 1 mg protein

- Requirements for analytical methods: Limits of detection (LOD) should be at lower ppm level (mg plant protein / kg meat product)

- Due to their high protein content lupine (36 – 48 % d.m.), pea (26 % d.m.), and soy (41 % d.m.) are often used for the production of emulsified sausages instead of pure meat (meat adulteration)

Selected marker peptides and corresponding target proteins

Marker peptide	Target protein	Species	Accession (NCBI)	Literature
QQEQQLEGELEK	Conglutin delta	*L. angustifolius*	P09931	
ISSVNSLTLPILR	Conglutin alpha	*L. angustifolius*	AEB33710	
NTLEATFNTR	Conglutin beta Vicilin-like protein	*L. angustifolius* *L. albus*	ABR21772 CAI84850	Wait et al., 2005
TLTSLDFPILR	Conglutin alpha	*L. angustifolius*	AAC49787	
ELTFPGSVQEINR	Convicilin	*Pisum sativum*	CAB82855	
LSSGDVFVIPAGHPVAVK	Vicilin	*Pisum sativum*	P13918	
LTPGDVFVIPAGHPVAVR	Provicilin	*Pisum sativum*	P02855	
HFLAQSFNTNEDIAEK	Glycinin G4	*Glycine maxima*	CAB57802	Leitner et al., 2006
EAFGVNMQIVR	Glycinin G2	*Glycine maxima*	KHN10743	Heick et al., 2011
FYLAGNQEQEFLK	Glycinin G1/G2	*Glycine maxima*	KHN10744/KHN10743	

Final HPLC-MS/MS method after optimisation of protein extraction temperature and tryptic digestion time

Defatting (PLE)
Acetone; 2 cycles (30°C, 50 bar)

↓

Protein extraction and tryptic digestion
0,5 mL TRIS-HCl (1 M, pH 8,2) and 50 mg defatted material (2 h at 70°C);
2 µg trypsin and 100 µL extract; 18 h at 37 °C

↓

Solid phase extraction (SPE)
Strata-X; elution mit 0,5 mL 80% ACN

↓

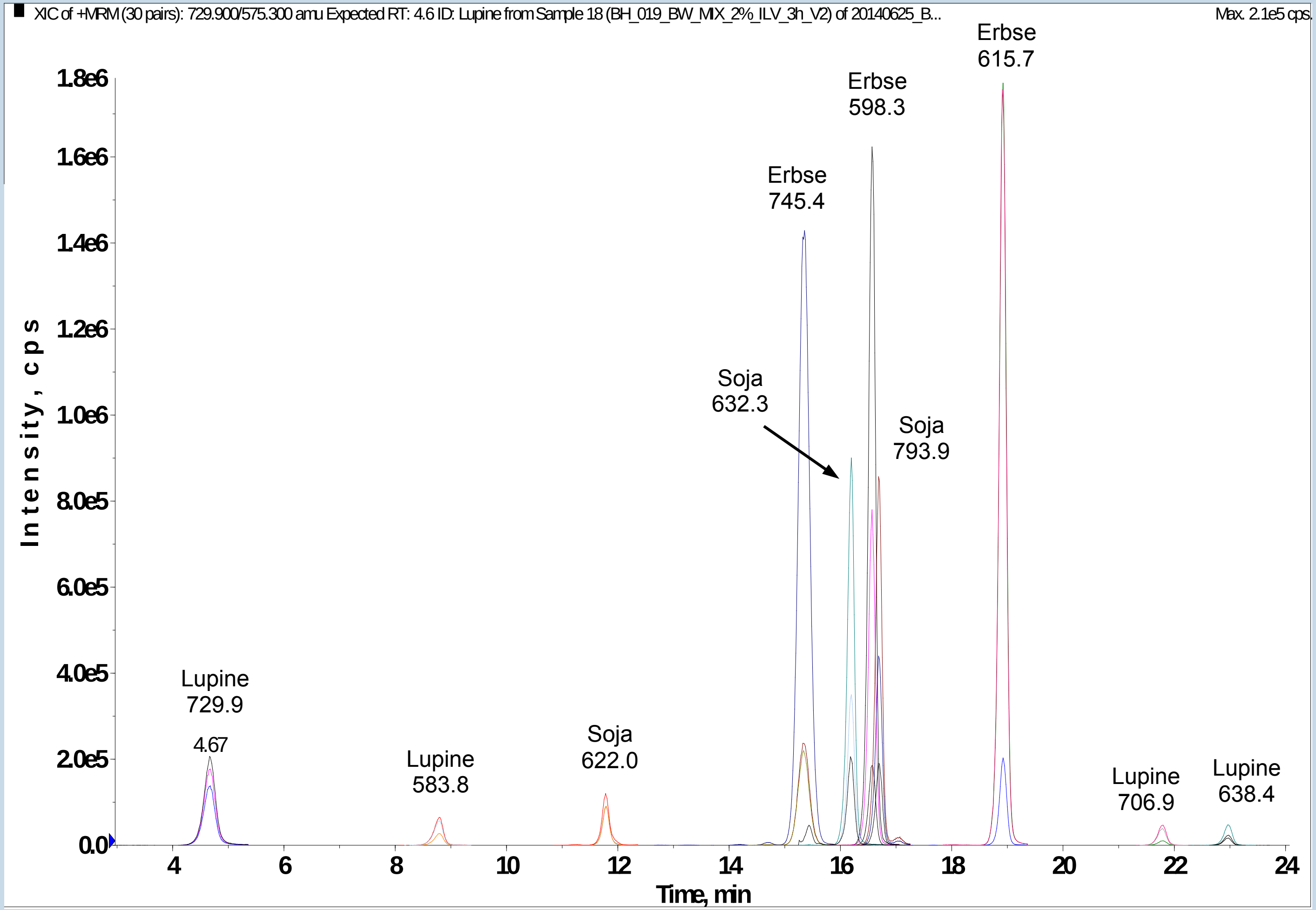
HPLC-MS/MS (QTrap 5500)
RP18-column; ACN/water-gradient; flow rate 0,25 mL/min;
source: 430 °C; ion spray: 5.5 kV; MRM-mode

Optimised HPLC-MS/MS-Method

Parameter of MRM-Mode (Multiple Reaction Monitoring)

Marker peptide (Target protein)	RT [min]	m/z (Charge)	Fragment ions	CE [V]
QQEQQLEGELEK (Lupine)	4.3	729.9 (+2)	575.3 (y5), 704.3 (y6), 817.4 (y7)	37/34/34
ISSVNSLTLPILR (Lupine)	22.2	706.9 (+2)	498.3 (y4), 712.5 (y6), 1026.6 (y9)	30/33/34
NTLEATFNTR (Lupine)	8.4	583.8 (+2)	838.4 (y7), 709.4 (y6), 951.6 (y8)	29/30/24
TLTSLDFPILR (Lupine)	23.4	638.4 (+2)	760.4 (y6), 1061.6 (y9), 960.6 (y8)	28/28/29
ELTFPGSVQEINR (Pea)	15.6	745.4 (+2)	999.5 (y9), 500.3 ($y9^{2+}$), 573.8 ($y10^{2+}$)	35/35/35
LSSGDVFVIPAGHPVAVK (Pea)	16.9	598.3 (+3)	875.5 (y9), 513.3 (y5), 988.6 (y10)	27/36/26
LTPGDVFVIPAGHPVAVR (Pea)	19.4	615.7 (+3)	903.5 (y9), 541.3 (y5), 1016.6 (y10)	30/36/30
HFLAQSFNTNEDIAEK (Soy)	11.8	622.0 (+3)	818.4 (y7), 919.4 (y8), 1033.5 (y9)	27/27/24
EAFGVNMQIVR (Soy)	16.4	632.3 (+2)	760.4 (y6), 916.5 (y8), 859.9 (y7)	30/34/30
FYLAGNQEQEFLK (Soy)	16.9	793.9 (+2)	283.1 (a2), 424.2 (b3), 638.7 ($y11^{2+}$)	45/37/34

Chromatogram of marker peptides in an emulsion-type sausage (addition of 2% flour resp. protein isolate)



Determined limits of detection (LODs) for lupine, pea, and soy

Sample	Pork [%]	Lupine flour (protein) [mg/kg]	Pea protein isolate (protein) [mg/kg]	Soy protein isolate (protein) [mg/kg]
0 --	49.1	0 (0)	0 (0)	0 (0)
1 --	49.1	1.28 (0.42)	1.28 (0.97)	1.28 (0.83)
2 ✓	49.1	6.4 (2.1)	6.4 (4.8)	6.4 (4.1)
3 ✓	49.1	32 (11)	32 (24)	32 (21)
4 ✓	49.1	160 (53)	160 (121)	160 (104)
5 ✓	48.9	800 (264)	800 (605)	800 (518)
6 ✓	47.9	4000 (1320)	4000 (3024)	4000 (2592)

⇨ LODs comparable to PCR- and ELISA-methods

Comparison of LODs with reference dose rates of the VITAL Expert Panel *(Taylor et al., 2014)*

⇨ Reference dose rates:
lupine: 4 mg protein
soy: 1 mg protein

⇨ LODs of the HPLC-MS/MS-method:
lupine: 2 mg protein/kg meat product
soy: 4 mg protein/kg meat product

⇨ Consumption of 100 g meat product:
lupine: 0,2 mg protein/100 g meat product
soy: 0,4 mg protein/100 g meat product

⇨ LODs for 100 g meat product are significantly below the reference doses

Conclusion

- By means of suitable and characteristic marker peptides (8 to 18 amino acids) from plant storage proteins a simultaneous detection of lupine, pea, and soy in meat products is reliably possible (LODs: lupine: 2 mg protein/kg, pea: 5 mg protein/kg; soy: 4 mg protein/kg)

- The use of at least three marker peptides for every legume protein with three mass transitions each enables a reliable detection of lupine, pea, and soy in meat products

- The method has high potential for the further development to a multi-method for the simultaneous detection of additional sources of foreign proteins

- Due to the achieved low limits of detection (low ppm range) a further development to a multi allergen screening method is conceivable