Antibiotics Residues in Honey:

Regulations and Methods of Analysis

Results, Conclusion and Outlook

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Company Profile of APPLICA

APPLICA is a highly specialised laboratory in the field of honey and honey products.

We are accredited as a testing laboratory in compliance with DIN EN ISO/IEC 17025, the internationally applied standard.

We operate worldwide on the basis of our innovative analytical methods, the latest technology and state-of-the-art equipment.

This has made us one of the world‘s leading laboratories for honey analyses.

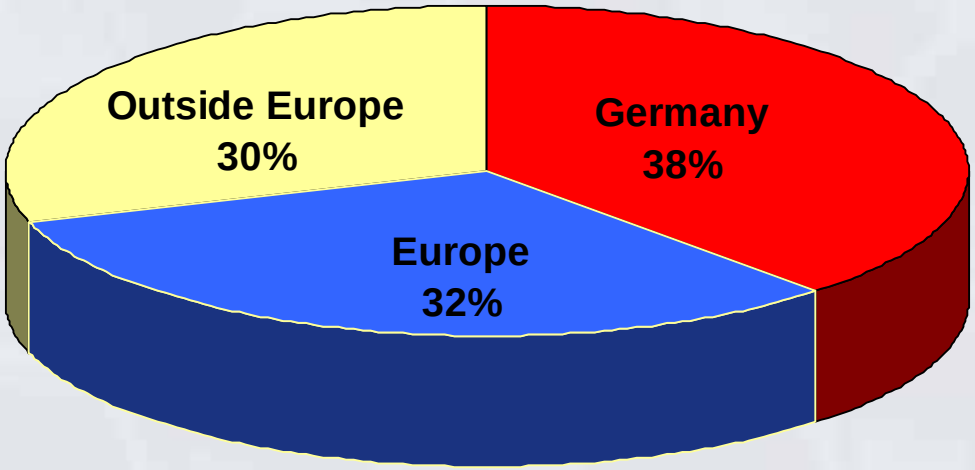
And APPLICA is maintaining and expanding its position even further by developing new methods and refining existing ones, by training its staff according to market needs, as well as by its various activities in research and development.

Roaming the vast field of honey analyses

Honey as a natural product is subject to especially strict regulations and controls. Our special analytical methods enable us to prove e. g. which

- Production and site conditions were available;
- Substances are contained in the product in detail;
- Prohibited and undesirable substances are present;
- Veterinary drugs have been used.

APPLICA has customers all over the world:



Residues in Honey

Regulations

2377/90/EC

... laying down a Community procedure for the establishment of
Maximum Residue Limits (MRLs)
of veterinary medicinal products in foodstuffs of animal origin

2002/657/EC

... concerning the
performance of analytical methods and the interpretation of results

2003/181/EC

... the setting of
Minimum Required Performance Limits (MRPLs)
for certain residues in food of animal origin

Residues in Honey

2377/90/EC

... laying down a Community procedure for the establishment of
Maximum Residue Limits (MRLs)
of veterinary medicinal products in foodstuffs of animal origin

Annex I

List of pharmacologically active substances used in veterinary medicinal products in respect of which maximum residue limits have been established

Annex II

List of pharmacologically active substances used in veterinary medicinal products for which it is not necessary for the protection of public health to establish a maximum residue limit

Annex III

List of pharmacologically active substances used in veterinary medicinal products in respect of which provisional maximum residue limits have been established

Annex IV

List of pharmacologically active substances which shall be prohibited throughout the Community for use in food-producing animals

Residues in Honey

2002/657/EC

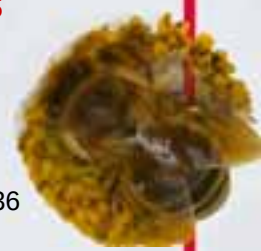
... concerning the
performance of analytical methods and the interpretation of results

It is necessary to ensure the quality and comparability of the analytical results generated by laboratories approved for official residue control.

This should be achieved by using quality assurance systems and specifically by applying of methods validated according to common procedures and performance criteria ...

It is necessary to determine common criteria for the interpretation of the test results ...

It is necessary to provide for the progressive establishment of Minimum Required Performance Limits (MRPLs) of analytical methods for substances for which no permitted limit has been established ...



Residues in Honey

2003/181/EC

... the setting of

Minimum Required Performance Limits (MRPLs)

for certain residues in food of animal origin

Chloramphenicol	**Honey**	**0.3 µg/kg**
Nitrofurane Metabolites	***(Honey)***	***1.0 µg/kg***

Residues in Honey

Methods:

- Reproducibility
- Linearity
- Limit of Quantification
- Limit of Detection
- Repeatability
- Sample Preparation
- System Performance

Laboratories:

- Competence and Experience
- Quality Control
- Technical Equipment

Determination of Veterinary Drugs in Honey

Analyte/s	Method	Confirmation Method	MRL MRPL	Limit of Detection
Amitraze	GC-MS		200 µg/kg	10 µg/kg
Chloramphenicol	ELISA Charm II Biocore Q	LC-MS/MS GC-MS	**0.3 µg/kg**	0.1 µg/kg
Coumaphos	LC-MS/MS GC-MS		100 µg/kg	0.1 µg/kg
Erythromycin	LC-MS/MS GC-MS			1 µg/kg
Fluvalinates	LC-MS/MS GC-MS			2 µg/kg
Nitrofurane Metabolites	LC-MS/MS		**0.5 µg/kg**	0.2 µg/kg
Streptomycin	LC-FLD Charm II ELISA	LC-MS/MS		5 µg/kg
Sulfonamides	LC-UV/FLD Charm II Biocore Q	LC-MS/MS		5 µg/kg
Tetracyclines	LC-UV Charm II	LC-MS/MS		5 µg/kg
Tylosin	LC-MS/MS			1 µg/kg

Determination of Nitrofurane Metabolites in Honey

History, Regulation, Method of Analysis, and Results

Regulation

Annex IV

List of pharmacologically active substances which shall be prohibited throughout the Community for use in food-producing animals

History

2002

At first, „no information to the public“ was necessary on Nitrofuranes in food acc. to the German authorities

Later this statement was altered regarding chicken and shrimps to „imports have to be tested for nitrofurane residues“

Methods for determining Nitrofurane Metabolites in food are being developed as: No tested methods or reference substances were available yet.

2003

Reference substances were found and an LC-MS/MS method for honey was developed with the following limit values:

Limit of Quantification: 1.0 µg/kg
Limit of Detection: 0.5 µg/kg

Nitrofurane Metabolites testing in honey then became routine. The analytical method was revalidated to:

Limit of Quantification: 0.5 µg/kg
Limit of Detection: 0.2 µg/kg

2004

Fine-tuning of the method based on the experience made with honeys of different origins.

Method of Analysis

Sample Preparation

1.0 (± 0.05) g of homogenised honey

50 µL Internal Standard (5 µg/kg) d4-AOZ / d5-AMOZ

5 mL of 0.1 M hydrochloric acid

0.1 mL of 2-nitrobenzaldehyde (0.05 M in methanol)

Incubation overnight (16 h minimum) at 37 °C

Neutralization with 0.42 mL of 1 M sodium hydroxide

Extraction with 5 mL of ethyl acetate

Evaporation to dryness with a nitrogen stream

Reconstitution with 0.5 mL of water/methanol (9/1)

Method of Analysis

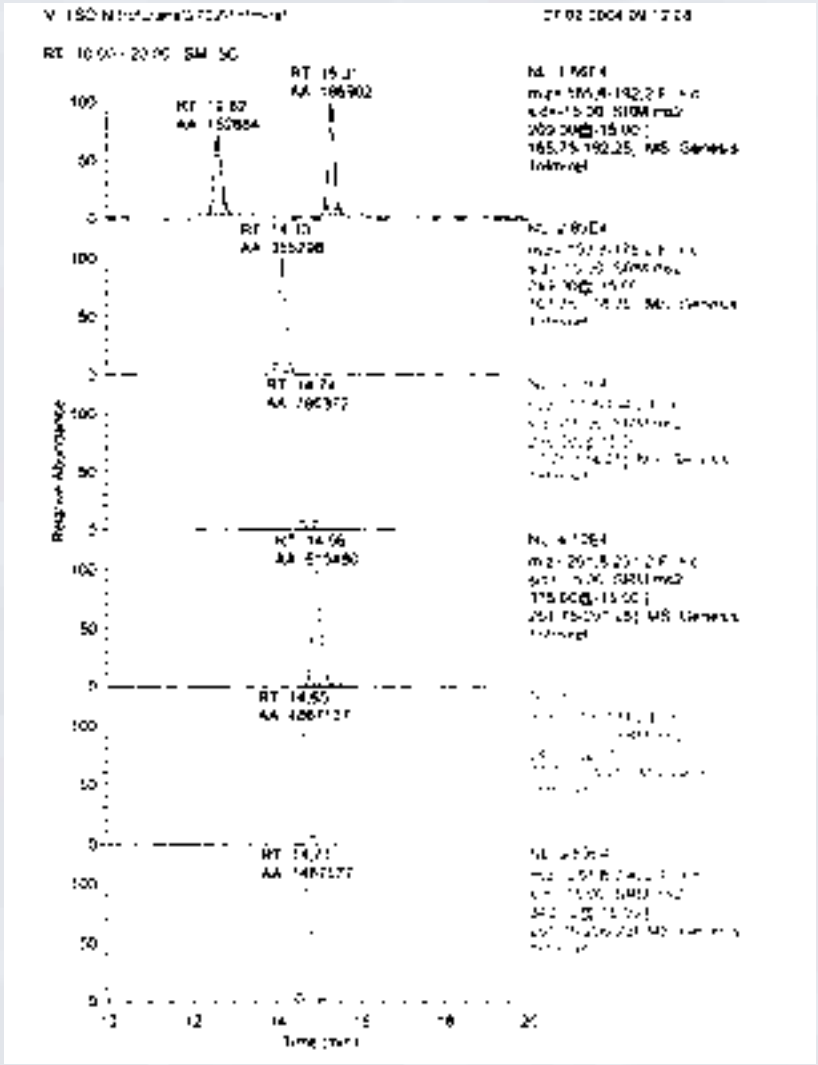
System - Thermo Electron LC-MS/MS Quantum Discovery

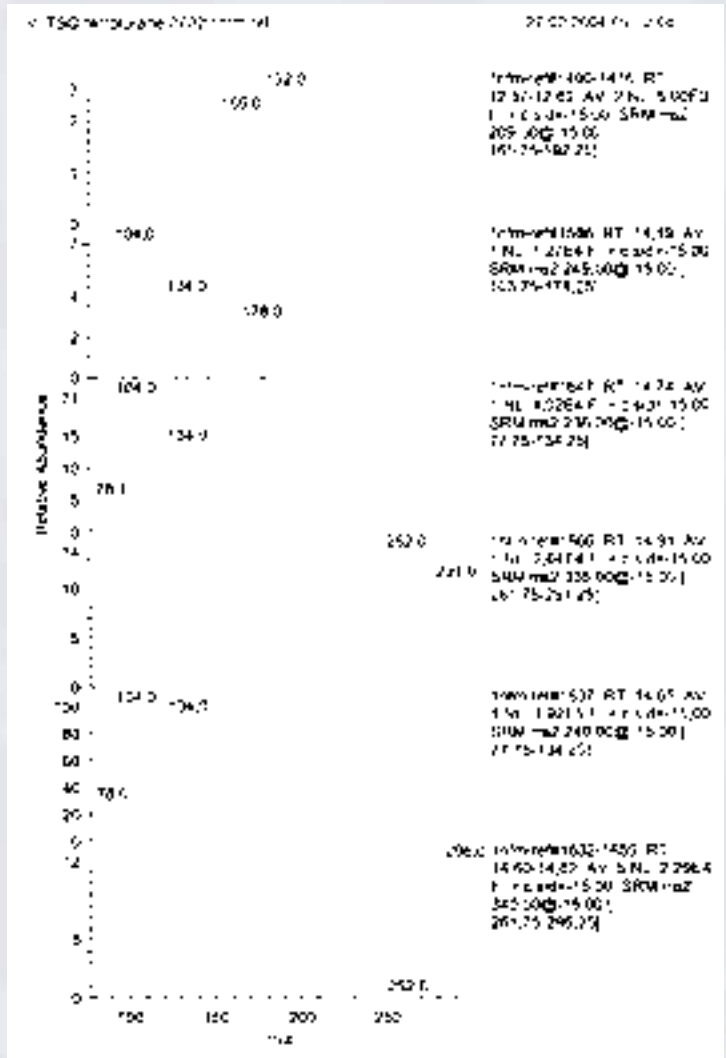
Separation Column	Phenomenex Synergi Polar-RP 125 x 2,0 mm ID; 4 µm
Eluent	Gradient Water / Methanol (0.01 % H_3C-COOH)

Inj. Volume:	20 µL	Flow:	0.2 mL/min
Run Time:	30 min		
Ionization mode:	ESI positive	Spray Voltage:	5000 VNitrogen
Flow:	55 Arb	Capillary Temp.:	300 °C
Collision Energy:	15 V		

Mass (SRM)	TSQ Parent	MS/MS-Product
SEM	209 $(M-H)^+$	166 / 192
AHD	249 $(M-H)^+$	104 / 134 / 178
AOZ	236 $(M-H)^+$	78 / 104 / 134
AMOZ	335 $(M-H)^+$	262 / 291
AOZ-d4	240 $(M-d4-H)^+$	78 / 104 / 134
AMOZ-d5	340 $(M-d5-H)^+$	267 / 296

Nitrofurane Metabolites in Honey





Quantification of Nitrofurane Metabolites in Honey

Reference: 1 µg/kg each

	SEM		AHD		AOZ		AMOZ	
	TSQ	LCQ	TSQ	LCQ	TSQ	LCQ	TSQ	LCQ
Variation Coefficient	3.22 %	8.98 %	6.53 %	10.83 %	3.24 %	3.52 %	3.63 %	7.88 %
Repeatability	0.09 µg/kg	0.25 µg/kg	0.18 µg/kg	0.31 µg/kg	0.09 µg/kg	0.10 µg/kg	0.10 µg/kg	0.22 µg/kg
Confidence	0.03 µg/kg	0.09 µg/kg	0.07 µg/kg	0.11 µg/kg	0.03 µg/kg	0.04 µg/kg	0.04 µg/kg	0.08 µg/kg

Quantification of Nitrofurane Metabolites in Honey

Contaminated Honey Samples

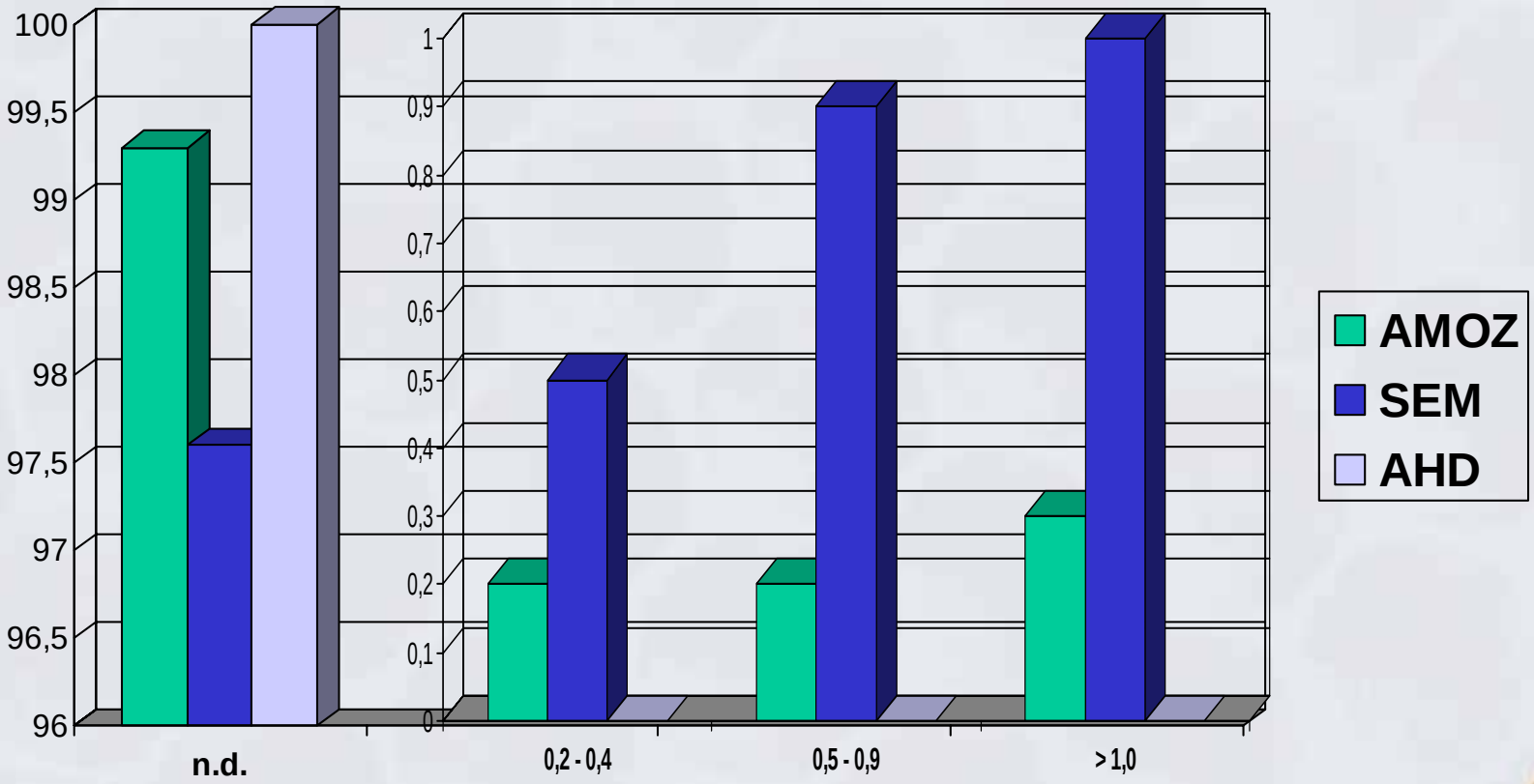
	AOZ		AOZ	
	TSQ	LCQ	TSQ	LCQ
Concentration	0.54 µg/kg	0.47 µg/kg	1.51 µg/kg	1.39 µg/kg
Variation Coefficient	10.15 %	14.25 %	3.24 %	13.30 %
Repeatability	0.15 µg/kg	0.19 µg/kg	0.14 µg/kg	0.52 µg/kg
Confidence	0.06 µg/kg	0.07 µg/kg	0.05 µg/kg	0.19 µg/kg

Nitrofurane Metabolites in Honey - RESULTS

July 2003 to September 2004 - AOZ

Nitrofurane Metabolites in Honey - RESULTS

July 2003 to September 2004 - AMOZ / SEM / AHD



Migration of Semicarbazide

2004/1/EC

... as regards the suspension of the use of azodicarbonamide as blowing agent

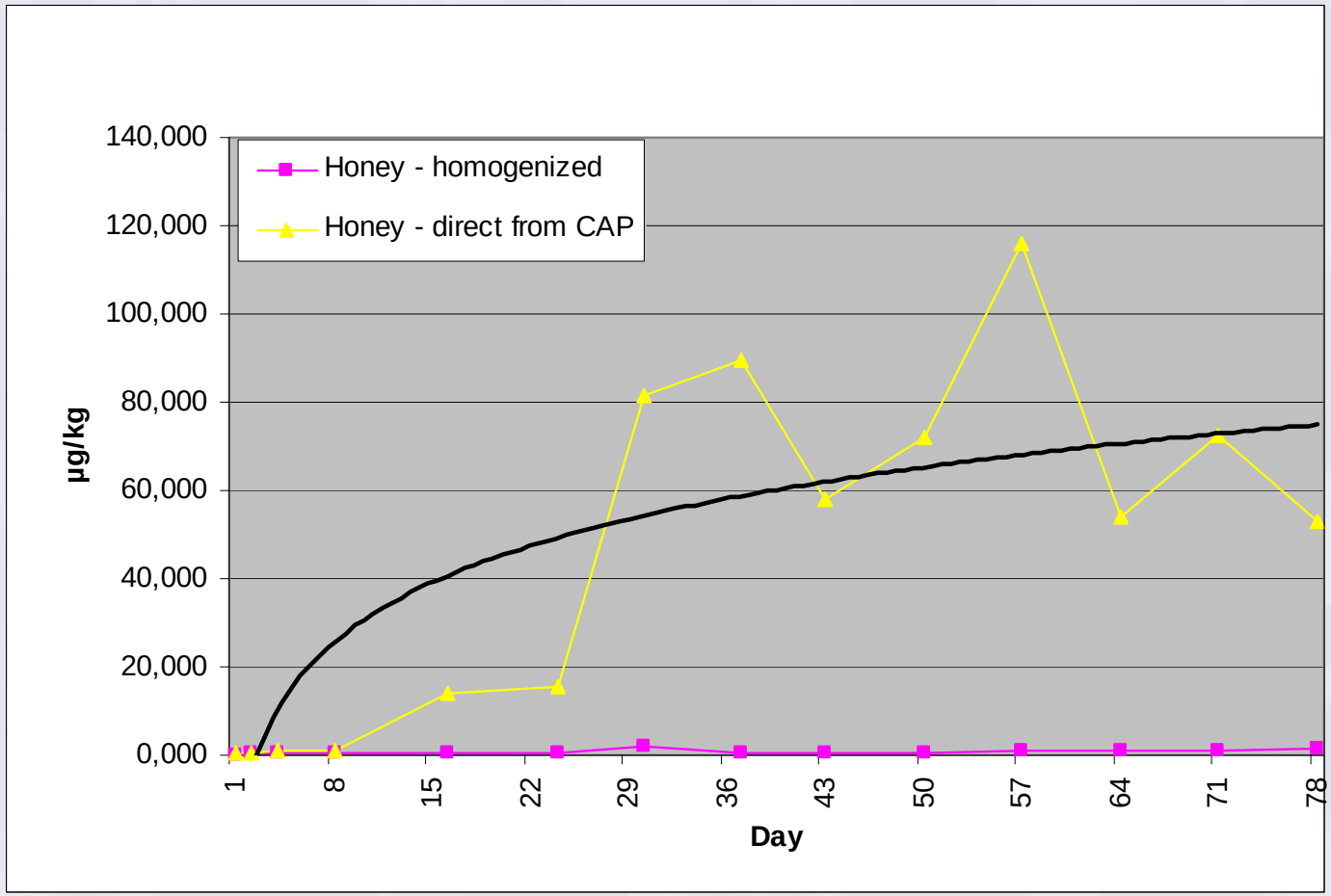
(2)

Azodicarbonamide is used as blowing agent in the manufacture of plastic gaskets in metal lids used for the closure of glass jars. New findings have shown that azodicarbonamide decomposes into semicarbazide (SEM) when heated during production of the foamed gasket and during sterilisation of the sealed glass jar.

Article 1

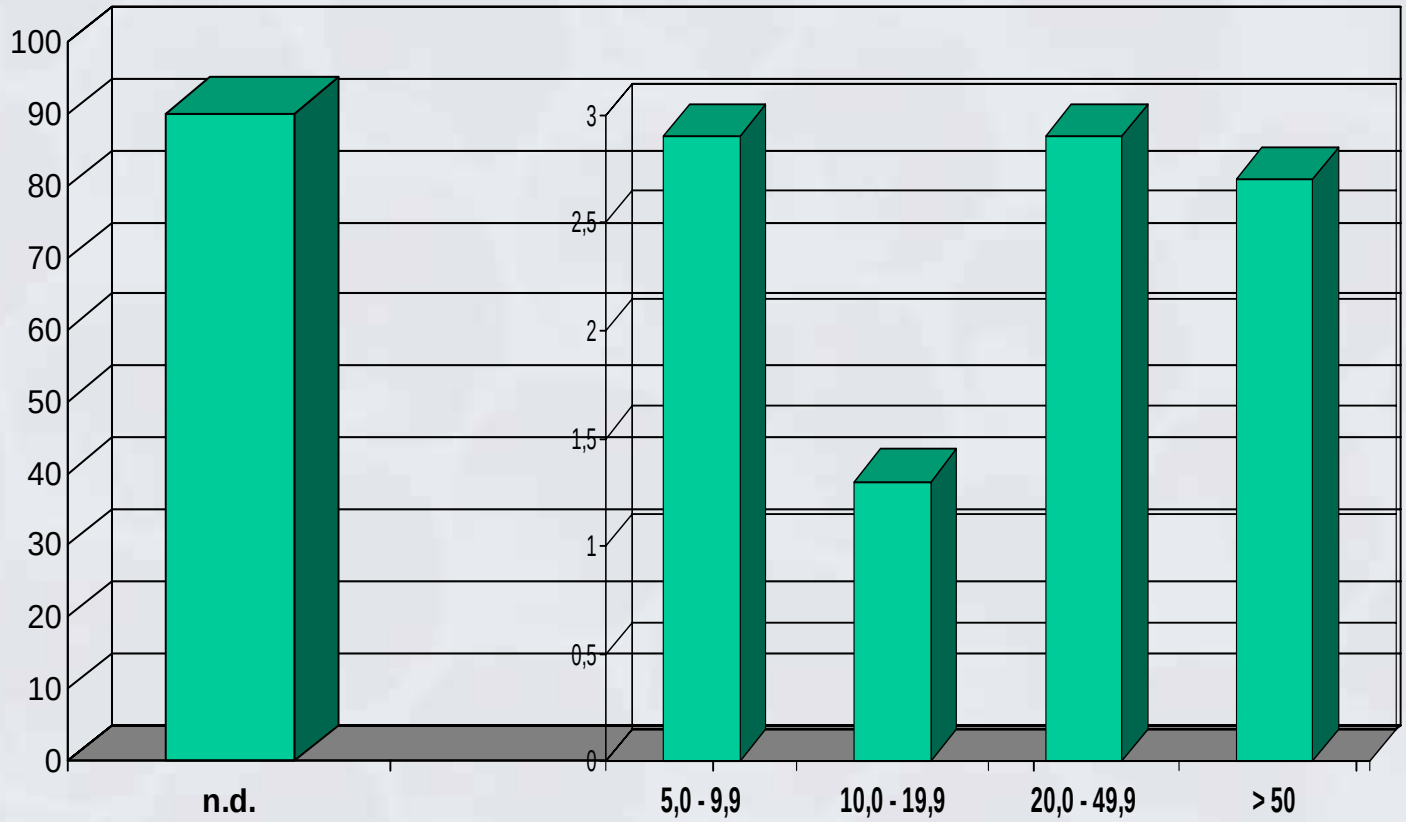
**‚For use only as blowing agent.
Use prohibited as from 2 August 2005.‘**

Migration of Semicarbazide



Residues Analysis on the example of:

Tylosin



Proposals,

Key Issues, and

Action to be Taken

Limits of Residues Analysis

Not known, not listed or not defined → **Zero Tolerance ?**

Different countries - different methods - different limits

Regulations are necessary for the international market to reach

Harmonisation
and
Internationally Accepted Regulations

Cooperation between Laboratories: Sulfochloropyridazine

Charm II - positive

↓

Confirmation with LC for known sulfonamides - negative

↓

Wrong positive ???

Sulfonamides - Comparison of HPLC and Charm II Results

Sample	Origin	Sulfonamide (HPLC) [µg/kg]		Charm II [cpm] *	Controls (Charm II) **
1	Bulgaria	-	-	5704	(+) 1440
2	"	-	-	5331	(--) 5499
3	"	-	-	5687	
4	"	-	-	5368	
5	"	Sulfathiazole	26	2317	
6	"	Sulfathiazole	26	1726	
7	Argentina	Sulfamethazine	17	957	(+) 1675
8	"	Sulfamethazine	24	859	(--) 5146
9	"	Sulfamethazine	65	653	
10	"	-	-	3779	
11	"	-	-	5841	
12	"	-	-	6533	
13	"	-	-	5166	
14	Mexico	-	-	4002	(+) 1494
15	"	-	-	4779	(--) 4406
16	"	Sulfathiazole	6	5041	
17	"	Sulfamethazine	7	5540	

* cpm = counts per minute

** (+) = positive control: negative honey spiked at 10 µg/kg sulfamethazine (SMZ)

(--) = negative control: negative honey

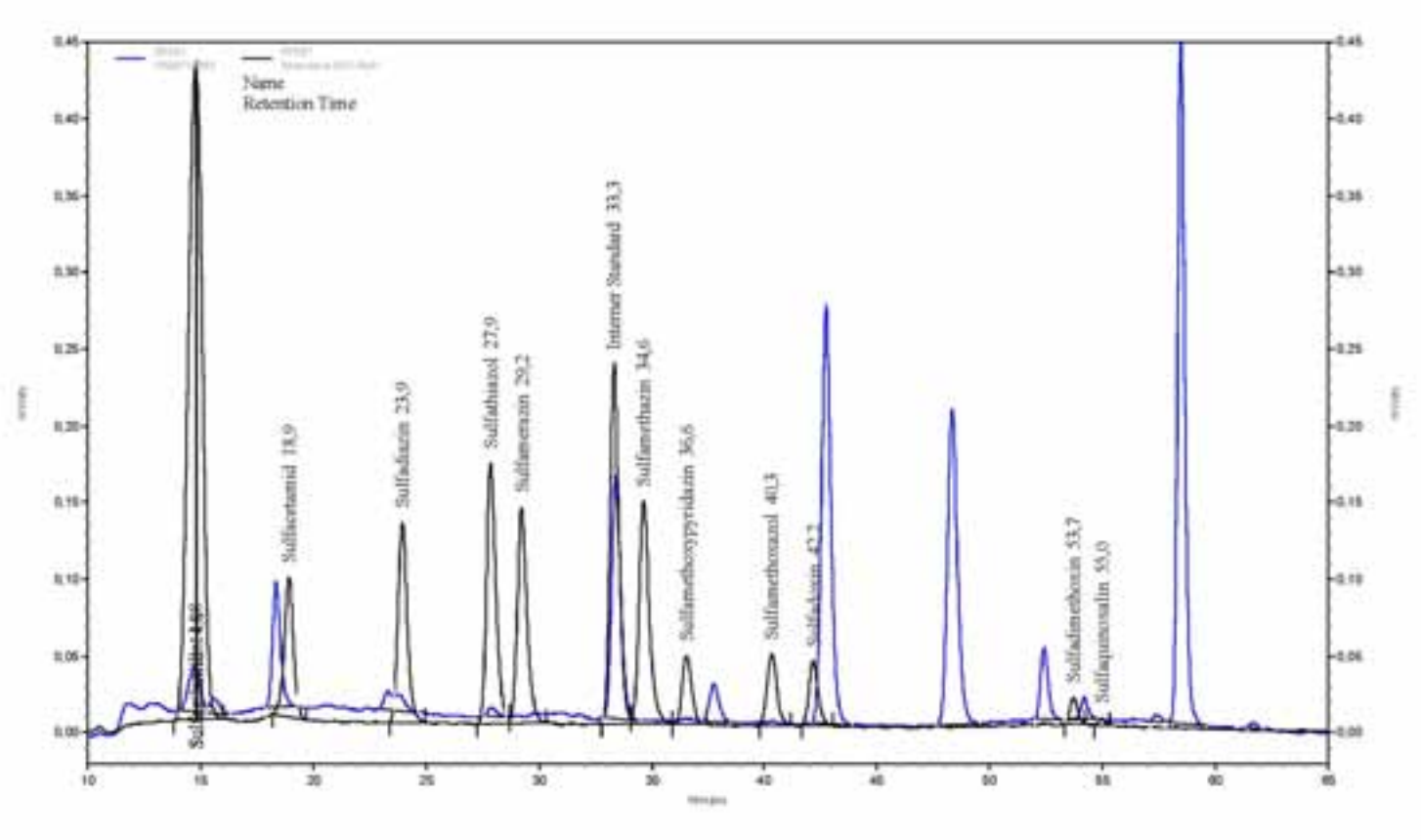
Control point: 2806 cmp ➡ value > 2806 cpm = negative Charm II result

➡ value < 2806 cpm = positive Charm II result

Sulfonamides in Honey

blue: Sample No. 294

black: Standard, 12 Sulfonamides 50 µg/kg



Cooperation between Laboratories: Sulfochloropyridazine

Charm II - suspicion of sulfochloropyridazine

↓

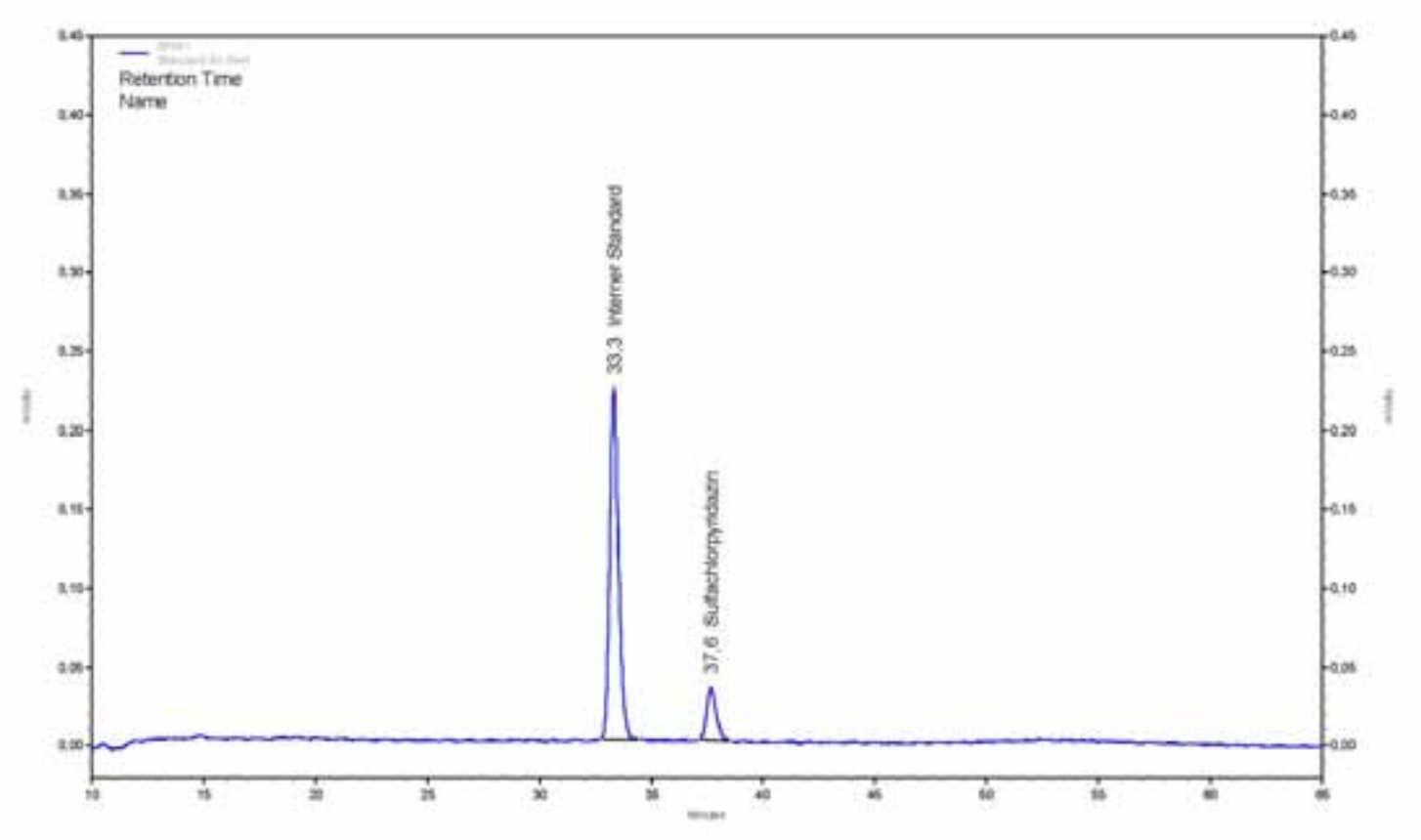
Confirmation with LC-MS/MS - positive

↓

Addition of sulfochloropyridazine to the group of sulfonamides for routine analysis by LC !

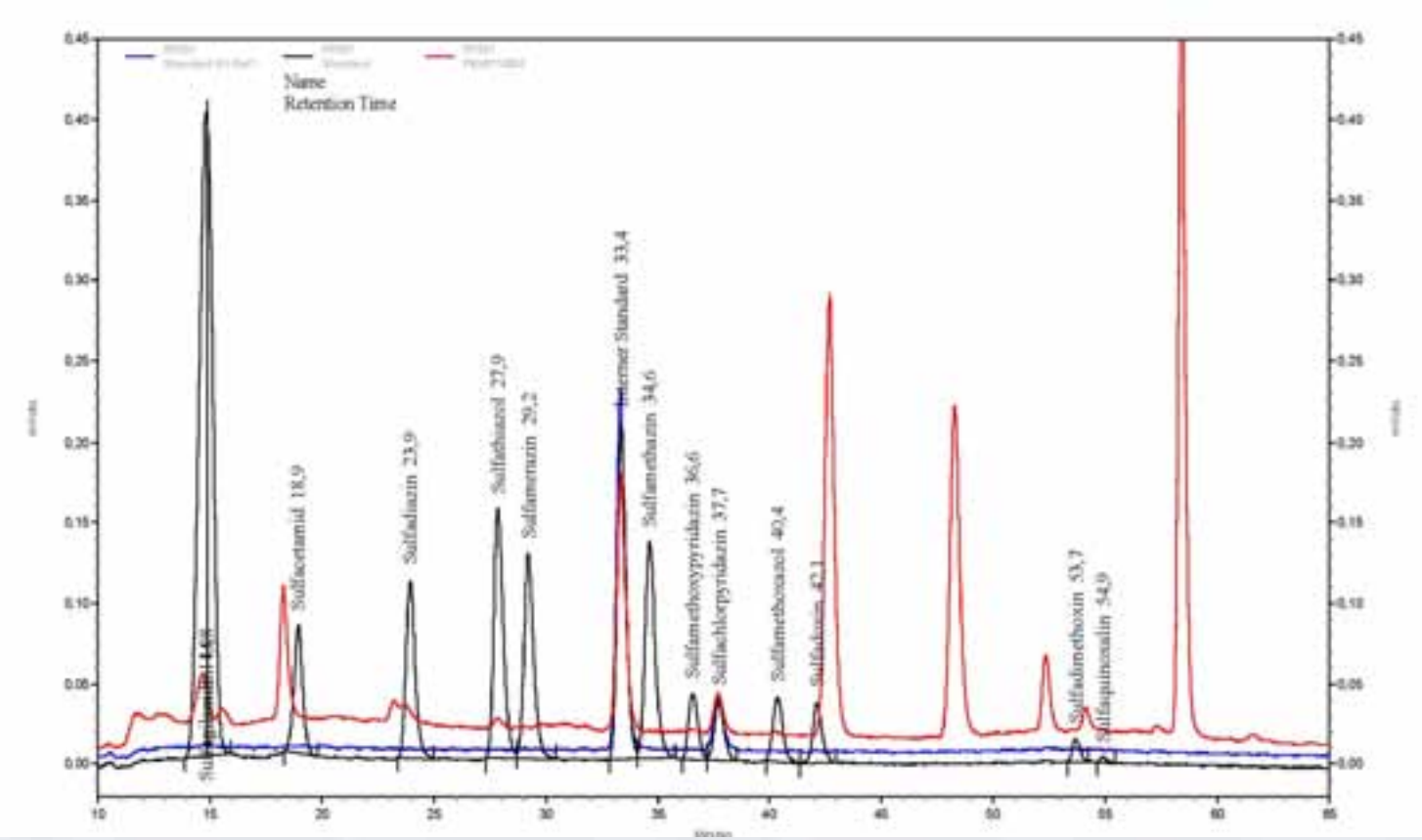
Sulfonamides in Honey

blue: Standard, Sulfachloropyridazine 50 µg/kg

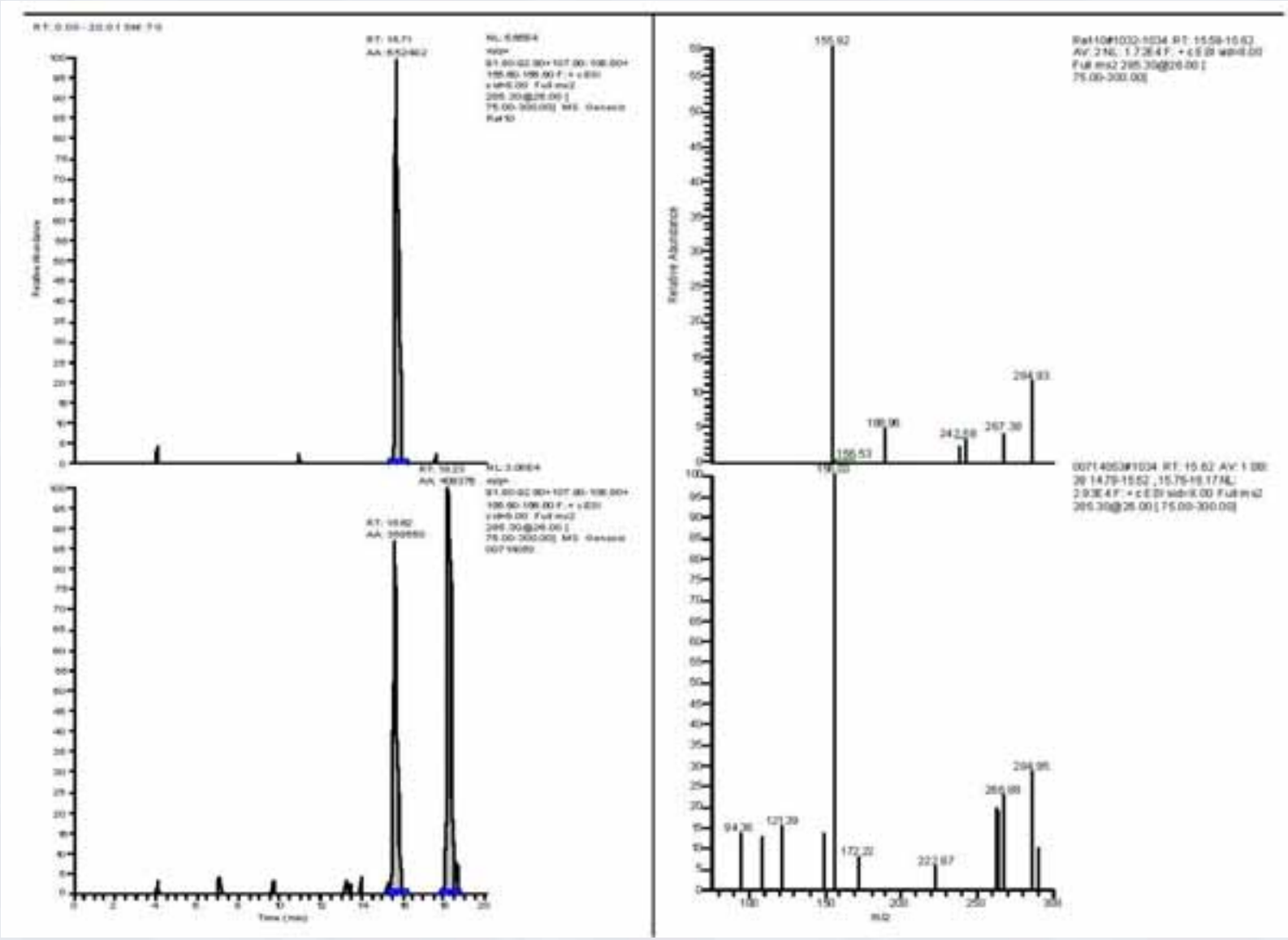


Sulfonamides in Honey

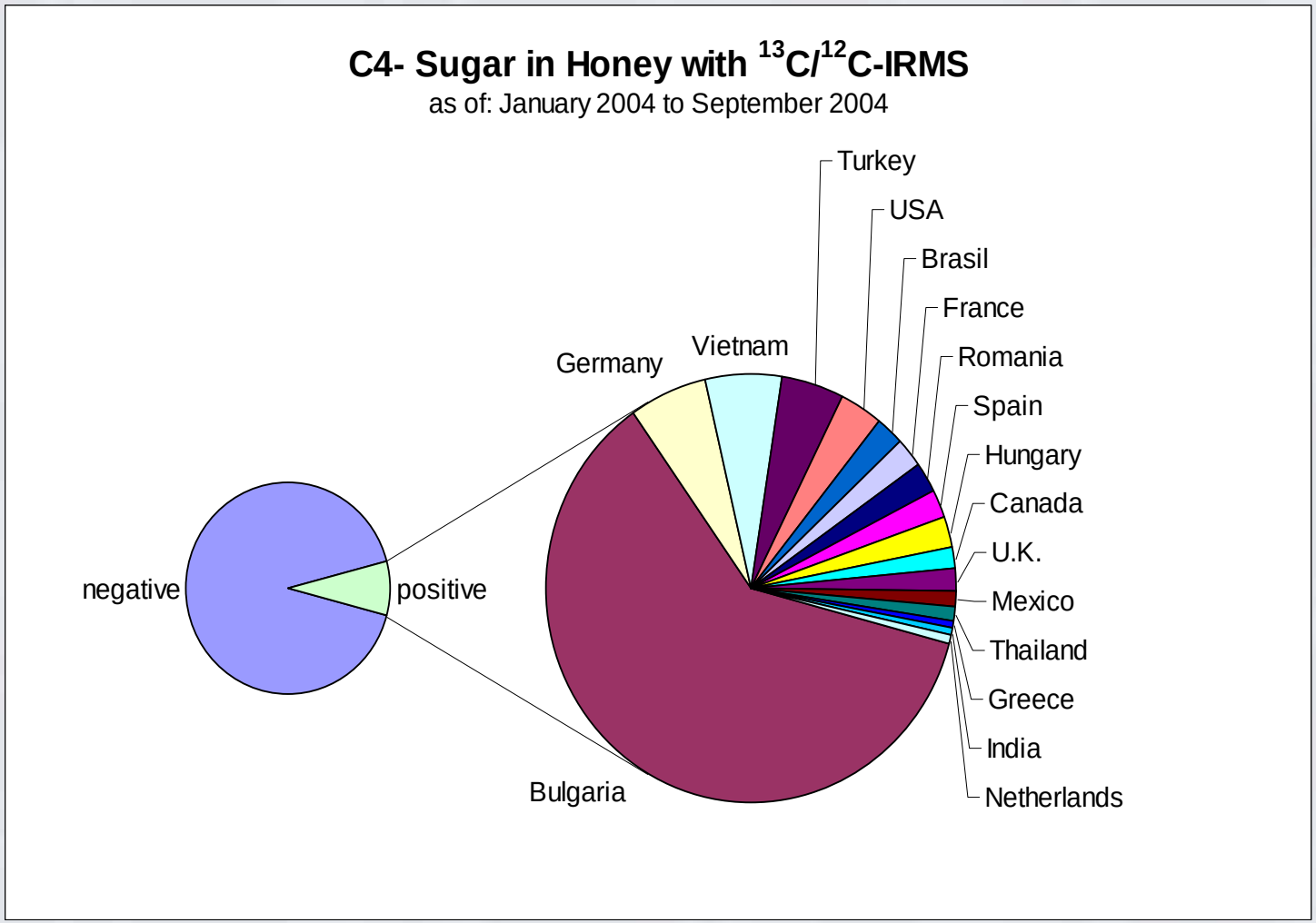
blue: Standard, Sulfochloropyridazine 50 µg/kg
black: Standard, 13 Sulfonamides
red: Sample No. 294



LC/MS of Sulfochlorpyridazine - Sample No. 294



Cooperation between Customers and Laboratories: Creation of a MAP for Residues in Honey

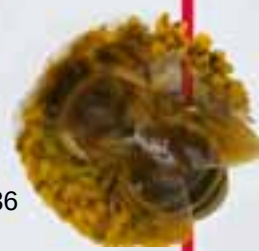


Cross-sectoral Cooperation: How to ensure quality

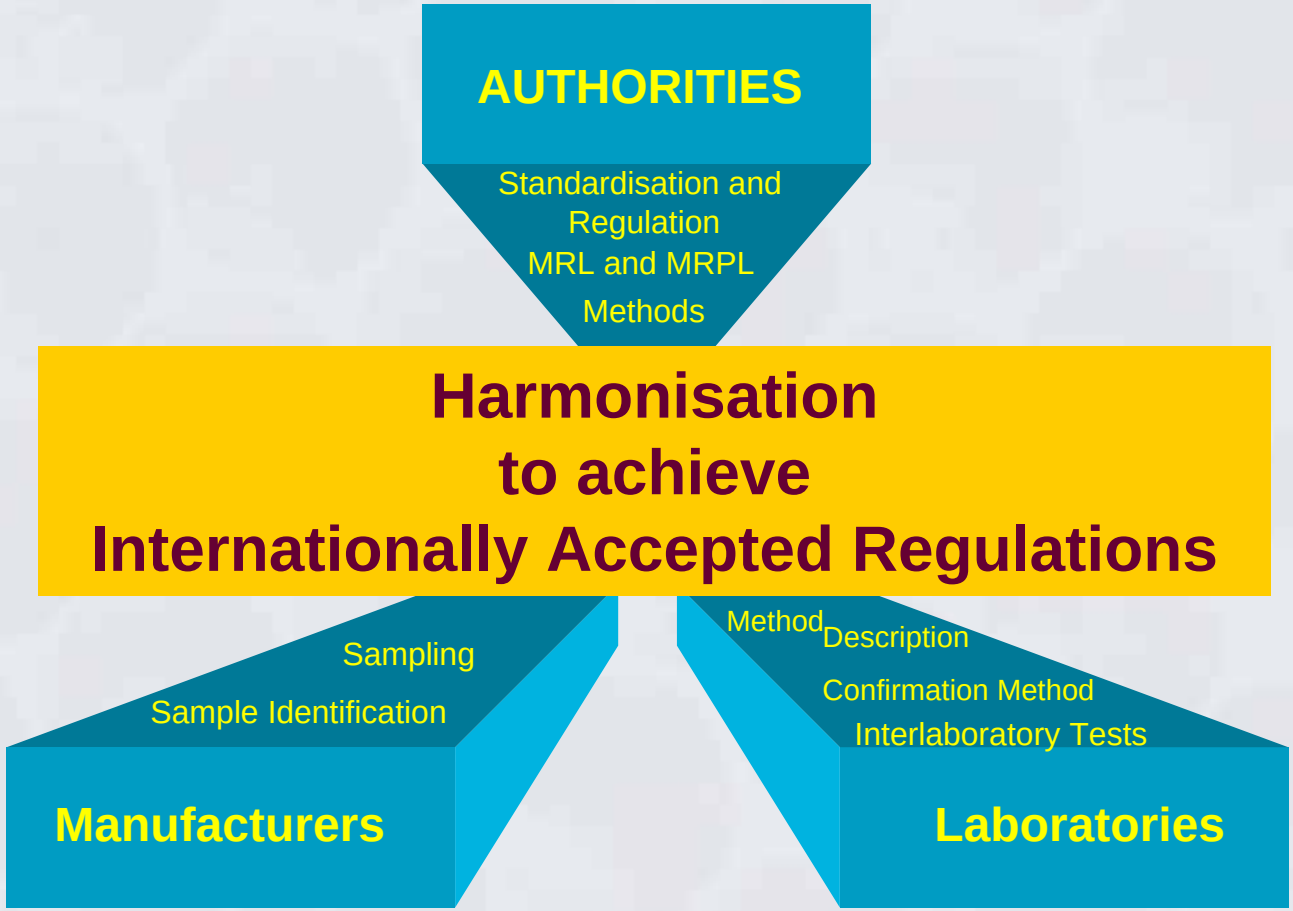
- Quality as the primary target for reliable analytics
- Producing reproducible results by proper sample preparation
- Using state-of-the-art technical equipment for testing
- Determining officially accepted methods and limit values
- Ensuring cross-border comparability of results
- Precondition for competent and experienced laboratories to get accredited by official bodies and institutes
- Harmonising different legislations in different countries and fixing internationally acceptable quality standards
- Setting action levels and evolving flexible quick-action strategies and procedures for covering new demands in the vast field of honey analytics in an ever changing environment
- Establishing a Rapid Alert System (RAS) for new and important findings

Cross-sectoral Cooperation: How to enhance enforcement

- Preparing the ground for creating a keen sense of importance of food safety in the official corridors of power on a local, state, national and international level
- Participating in a brisk and open-minded exchange of relevant news, views and ideas between industry, laboratories and authorities
- Giving Public Relations a more prominent role to ensure that not only problems but also achievements are communicated in public, thus strengthening our interests from the outside
- Speeding up solutions, agreeing on interpretation of results and making them internationally accepted by all parties involved
- Keeping a closer eye on quality control and adherence to rules and regulations, codes and standards to restore confidence in the product honey



Conclusion and Outlook



My thanks go to:

- ***You, as the audience, for your attention and your interest shown in my lecture***
- ***The APPLICA cooperation partners and customers***
- ***All my staff members who participated in the elaboration of the analytical results:***