Two approaches on food safety along the poultry chain – analysis of antibiotic residues in poultry meat and species authentication

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Introduction

Today the European consumer is offered a wide range of safe and high quality products. On the other hand food safety problems have become a frequently recurring problem during the recent years. This is also due to never-ending food scandals such as BSE (2000), nitrofene in ecological and conventional produced cereals (2002) and the "chloramphenicol scandal" (2002). At the same time food production chains are becoming increasingly complex. Every link in the chain must be as strong as the others, if the health and well-being of the consumer is to be adequately protected. This includes developing a system to link products to their source, identify components, counteract frauds and insure that proper processing has taken place. Fraud detection includes the ability to authenticate with respect to food labelling as well as compliance with ingredient composition.
There again, to address the consumers need for the highest possible reliability with regard to food of animal origin analytical methods are needed that are fast, accessible and reliable in detection of residues and contaminants.
However, at the foundation of any endeavour toward food safety should be a solid understanding of the interrelation and complexity of the food chain itself.
This paper will, taking the poultry production chain as an example, describe two approaches to key issues of food safety – the absence of residues and contaminants as well as the authentication of the end-product.

Traceability within food chains

The General Food Law covers the entire supply chain [Regulation (EC) 178 (2002), Article 18, paragraph 1]. In order to be able to trace products and retrieve related information, producers must collect information and keep track of products during all stages of production. Traceability can be divided into two key functions, tracking and tracing. Tracking is defined as the ability to follow the path of an item as it moves downstream through the supply chain from beginning to end. Tracing is the ability to identify the origin of an item or group of items, through records, upstream in the supply chain. Methodologies for the analysis of food and feed materials combined with information technology systems are essential to delivering a working tracking and tracing system (Schwägele, 2005).
A working tracking and tracing system as well as a substantial knowledge about the interdependencies within the food chain is exactly what is needed to deal successfully with potential vulnerabilities.
The specific targeted research project ΣChain: "Developing a Stakeholders´ Guide on the vulnerability of food and feed chains to dangerous agents and substances" (FP6 – 518451) addresses existing as well as potential vulnerabilities of food chains using four "vulnerable" products (drinking water, milk powder, poultry meat and farmed salmon) as case studies. In each of the four cases, the chain will be reviewed from start to end, links will be mapped and the contamination potential at each link of the chain will be evaluated.

The clear emphasis is on full chain traceability from food to farm. Especially transfer points, where responsibilities for the product changes were considered as vulnerable. Furthermore particular attention will be paid to cross border/ world wide chain integrity.

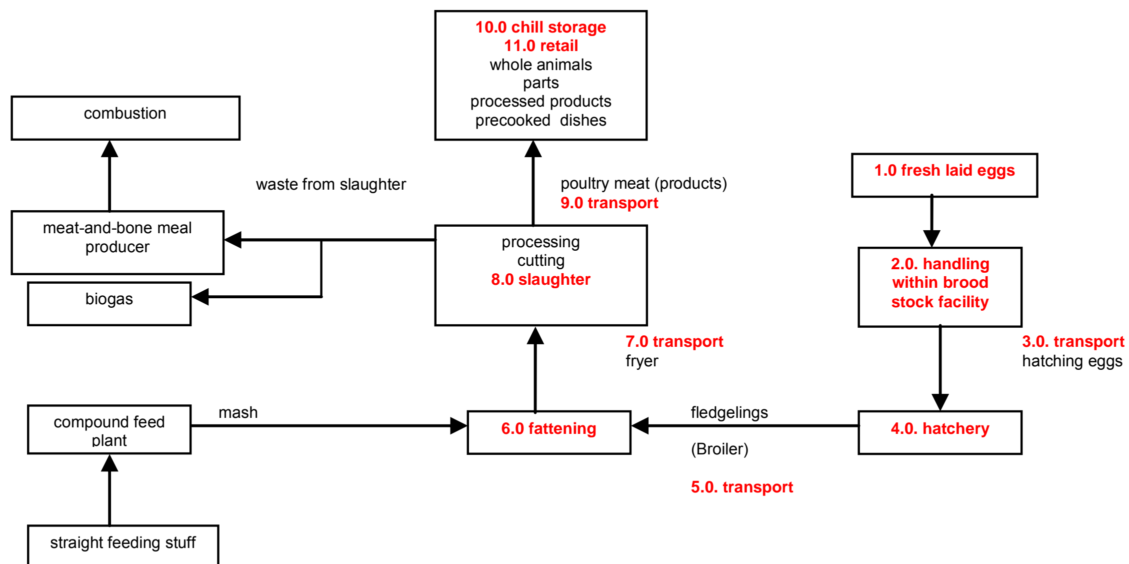

Fig. 1: Generic poultry production chain [according to: von Bittner, G and Windhorst, H (2005)]

Figure 1 shows a – very generic – example for a poultry production chain including the impact of animal feed on the chain. This is important because all of the most serious consumer food scares in the last ten years have been attributed to contaminated feeds going into food of animal origin.
With respect to traceability along the full supply chain of meat and meat products the following aspects are of importance. They shall, if possible, give information on cross contamination or carry over in food and feed, animal species, origin, authenticity, age, composition and production system (including feed).
In the following two approaches to retain food safety will be examined more closely: veterinary drug residues, esp. sulfonamides in food of animal origin, probably via cross contamination or carry over, and the authenticity and compliance with ingredient composition by species authentication.

Veterinary drug residues – EU Legislation and regulation
Highly relevant in respect to safety of food of animal origin are residues of veterinary drugs. The use of veterinary drugs within the European Union is regulated by means of the Council Regulation (EEC) No. 2377/90 describing a procedure for the establishment of maximum residue levels (MRLs) for veterinary medicinal products in foodstuff of animal origin including meat, fish, eggs and honey. Its annexes present substances, for which MRLs have been established (Annex I), substances, for which it is not considered necessary to establish MRLs (Annex II), substances with provisional, temporary MRLs (Annex III) and substances, which are not allowed to be used for food producing species (Annex IV). The prohibition of the use of growth promoting substances such as hormones or ß-agonists is established with Council Directives No. 96/22/EC and 2003/74/EC. Since January, 1st 2006 according to Regulation (EC) No. 1831/2003 the use of antibiotic growth promoting substances as additives for use in animal nutrition is forbidden.

While Council Directive No. 96/23/EC defines measures to monitor certain substances and residues thereof in live animals and animal products it divides veterinary drugs into two groups: group A covering prohibited substances in compliance with the Annex IV of the Council Regulation (EEC) No. 2377/90 and group B containing agents, in compliance with Annexes I and III of the Council Regulation (EEC) No. 2377/90. Commission Decision 2002/657/EC establishes criteria and procedures for the validation of analytical methods for the detection of residues. For substances according to Annex IV of the Commission

Regulation (EC) No. 2377/90 Commission Decision 2003/181/EC defines minimum required performance limits (MRPLs) for the determination of their residues in food of animal origin.

Determination of sulfonamide residues in poultry meat

Aim of the current study was the implementation of an analytical method for the determination of sulfonamide residues within poultry meat. To realise this, a simple and fast sample preparation method was elaborated. High performance liquid chromatography (HPLC) was used to separate the extracted compounds. Within the scope of method optimisation two detection techniques were applied: diode array detection (DAD) as well as fluorescence detection (FLD, following pre-column derivatisation). Residues in food are of concern due to their carcinogenic nature and the possibility to develop antibiotic resistances within humans. EU regulatory bodies have set maximum residue levels (MRL) for muscle, fat, liver, kidney and milk at 100 µg/kg, defined as sum of all parent substances present within the sample [Council Regulation (EEC) No. 2377/90].

Materials and Methods

For this study a selection of commonly used sulfonamide antibiotics and coccidiostatics were chosen. Sulfadimethoxine (SDX), sulfamethazine (sulfadimidine) (SMZ), sulfaquinoxaline (SQX), sulfamethoxazole (SMX), sulfadiazine (SDZ), sulfamerazine (SMR), sulfamethoxypyridazine (SMP), sulfachloropyridazine (SCP) and sulphanilamide (SA) were obtained from Dr. Ehrenstorfer GmbH, Augsburg, Germany. Sulfacloropyrazine sodium (sulfaclozine) (SCL) was obtained from Chemos GmbH, Regenstauf, Germany. All chemicals were analytical or liquid chromatographic (LC) grade and were purchased from Merck KGaA, Darmstadt, Germany. Trifluoroacetic acid (TFA) was for protein sequence analysis and also from Merck KGaA, Darmstadt, Germany. Flurescamine (Fluram®) was purchased from Sigma-Aldrich, Steinheim, Germany. The stock solutions were prepared in Methanol (c = 1 mg / mL) except sulfaquinoxaline (SQX) which was dissolved in acetonitrile (c = 0,5 mg/mL). The desired concentrations of working standard solutions were adjusted by diluting the stock solutions with the mobile phase acetonitrile-water (5/95 v/v; the water containing 0,1 % TFA) for diode array detection (DAD) and acetate buffer for fluorescence detection (FLD) respectively.

The Beckmann Coulter System Gold HPLC systems consisted of programmable solvent delivery modules 126 and 126NM, autosampler 507 and 508, column thermostats from Beckmann Coulter and Jet stream 2Plus, respectively, diode array detector modules 168 and 168NM as well as of the Shimazu fluorescence detector RF-10A XL. The chromatographic separation was carried out on a Chromolith Performance RP-18e, 4,6x100 mm combined with a guard column Chromolith Performance RP-18e, 4,6x10 mm, Merck KGaA, Darmstadt, Germany. The chromatographic conditions for both methods are described in table 1.

Chicken and turkey meat for control and recovery experiments were purchased at local markets. Sample preparation was done using a modification of the protocol described by Posyniak et.al. (2005). The samples were minced, mixed thoroughly and deep frozen until analysis. An accurately weighted amount of 5 g was placed into a 30 mL centrifuge tube. For recovery experiments working standard solutions were added to the tissues at appropriate microliter aliquots.

Table 1: HPLC conditions, separation and detection of sulfonamide residues using DAD and FLD

Detection	DAD		FLD	
Solvent A	0,1% TFA in water		0,1% TFA in water	
Solvent B	acetonitrile		acetonitrile	
Gradient	time (min)	%B	time (min)	%B
	0,0	5	0,0	25
	20,5	40	15,0	25
	25,5	40	30,0	30
	30,5	5	35,0	80

	35,5 5	40,0 80 45,0 25 50,0 25
Flow rate	2,5 mL/min	2,5 mL/min
Temperature	30°C	30°C
Injection	20 µl	10 µl
Detection conditions	270 nm, 245 nm, 205 nm	Excitation: 405 nm, Emission: 495 nm

These samples were allowed to stand for at least 30 min at room temperature. Five mL acetonitrile were added and the sample was homogenised with ultra turrax for 1 min. 500 mg sodium sulfate anhydrous were added, the sample was mixed again for several seconds and allowed to stand for 10 min.
The mixture was centrifuged at 4000 rpm for 10 min. A 2 mL aliquot of the acetonitrile phase was transferred into a 2 mL microcentrifuge vial containing 25 mg Bakerbond Octadecyl. The resulting mixture was vortexed for 1 min and centrifuged at 5000 rpm for 5 min. The supernatant was separated from the solids and transferred into a fresh microcentrifuge vial. The resulting liquid was evaporated to dryness under nitrogen at 37 °C. The solid residue was dissolved in 400 µL acetonitrile-water (5/95 v/v; the water containing 0,1 % TFA) filtered and transferred into an autosampler vial for DAD measurement. For fluorescence detection the solid residue was dissolved in 400 µL acetate buffer, filtered and transferred into an autosampler vial. Derivatisation with Fluram® was carried out pre-column within the autosampler 508 for 30 min at 20 °C.

Results and Discussion
Most of the more classical approaches to determine sulfonamide antibiotic residues in meat samples involve DAD detection. Thus starting point of the current study was the separation of the target analytes using a monolithic RP18e separation column with subsequent DAD detection. In table 2 the performance parameters of this method are listed. The calibration curves were based on an external standard mixture. Each concentration was analyzed 3 times for defining the calibration curve (peak area/concentration). For the DAD method the analysis time is as fast as 10 minutes (35 minutes in total including equilibration). A base-line separation of all analytes with minimal interference of matrix peaks was achieved. UV spectra measurement was carried out and the spectra were matched with an existing UV spectra library.
To perform recovery experiments the control samples of chicken and turkey lean meat were spiked according to the procedure described above with different aliquots of working standard solutions, prepared and analysed. Taking into account recovery rates and blanks, the limit of quantification of the DAD method can be determined within the range of the MRL. Changing the detection limit from DAD to FLD (after pre-column derivatisation with Fluorescamine) leads to a hundredfold increase of sensitivity.

Table 2: Performance parameters, and detection of sulfonamides by DAD and FLD

Detection	DAD	FLD
Linear dynamic range of calibration ($R^2 \geq 0,99$)	500-10.000 ng/mL	5-1000 ng/mL
LOD calc. (S/N=3)	60 - 150 ng/mL	0,16 - 1,6 ng/mL
LOQ calc. (S/N=10)	300 - 450 ng/mL	0,5 - 5,5 ng/mL

R^2= coefficient of determination; LOD = limit of detection; LOQ = limit of quantification; S/N = Signal to noise ratio; calc.: calculated

Again the performance parameters of the FLD method are shown in table 2. Taking into consideration recovery rates and blanks, the limit of quantification of the FLD method can be determined within the range of the tenth part of the MRL. Here again a base-line separation of the analytes was achieved. Interferences with matrix peaks were at minimum.

Conclusions - Determination of sulfonamide residues in poultry meat

Two methods for the determination of sulfonamide residues in poultry meat were implemented. Both can be applied after a fast and not complicated sample preparation protocol for the determination of sulfonamide residues in meat samples. The fast DAD method having the advantage of the UV spectra available for confirmation lacks a low enough limit of quantification. The FLD method not having the excess value of identity confirmation, on the other hand, provides limits of quantification suitable for the determination of sulfonamide residues well below the MRL. In the near future a confirmation method using electrospray ionization/mass spectrometry in case of positive results within the screening will be established.

Authentication of domestic poultry species in meat and meat products

In recent years, DNA analytical techniques applying species specific PCR systems have been described for animal and plant species. These methods allow for the analysis of very complex samples with high sensitivity. Aim of the current study within the scope of ΣChain was the authentication of the most common domestic poultry species in Europe (chicken, turkey, duck, goose, pheasant, quail and guinea fowl) in meat and meat products and thereby to provide a method to detect adulteration. This was done by the adaptation and development of species-specific polymerase chain reaction (PCR) primer systems, respectively. Within this study the mitochondrial *cytochrome b gene* was applied for interspecies comparison.

Materials and Methods

Where available species specific primer systems were adapted from literature as follows: chicken (gallus gallus): (Dooley et. al., 2004), goose (anser cygnoides): (Colombo et. al., (2001) and duck (cairina moschata): Molspec-ID Online database (MSDB) 1.0 Entry No.: 22). The mitochondrial (mtDNA) sequences coding for *cytochrome b* for the species turkey (meleagris gallopavo), pheasant (phasianus colchicus), quail (coturnix coturnix) and guinea fowl (numida meleagris) were searched for in various genomic databases such as genbank (National Centre for Biotechnology Information, NCBI) and EMBL (European Molecular Biology Laboratory). using the Entrez Global Query Cross-Database Search system of NCBI and the Sequence Retrieval System (SRS) of EMBL.

Sequence alignment using CLUSTAL (multiple Sequence Alignment) as well as the cross checks against various nucleotide databases (Basic Logical Alignment Search Tool, BLASTN) were done using the HUSAR Sequence analysis package (Heidelberg UNIX Sequence Analysis Package W2H 4.1) of the German Cancer Research Centre. DNA was extracted from raw meat and processed meat products using a modified Hexadecyltrimethylammonium bromide (CTAB) protocol (Binke, 2004).

Meat samples and processed meat products containing the above mentioned species were purchased from local markets. PCR was carried out using the Thermocycler GeneAmp PCR System 9600 (Perkin-Elmer GmbH, Überlingen, Germany). The master mixture was composed as follows: 16,3 µL PCR-water, 2,5 µL PCR-buffer (tenfold concentrated, Qiagen, Hilden, Germany), 1,0 µL deoxynucleotide triphosphate (dNTP)-mixture (10 mM per dNTP, Qbiogene, Heidelberg, Germany), 1,0 µL per primer (forward and reverse, c = 10 µM, Operon, Köln, Germany, 1,0 µL MgCl2 (25 mM, Qiagen, Hilden, Germany) and 0,2 µL HotStarTaq™ DNA-polymerase (5 units/µL, Qiagen, Hilden, Germany). To this master mixture 2,0 µL DNA-extract (tenfold diluted with Tris-EDTA buffer 1:10) is added to a total volume of 25,0 µL.

Initial denaturation and the denaturation step was carried out at 95 °C for 15 min and 30 s, respectively, elongation temperature was chosen in accordance with the primers melting

temperatures, elongation took place at 72 °C for 30 s. The complete PCR-program was carried out for 27 to 30 cycles. Detection of PCR-products was accomplished using gel electrophoretic separation on polyacrylamide gels with subsequent visualisation using ethidium bromide.

Results and Discussion

For all of the above mentioned domestic poultry species species specific primer systems based on the mitochondrial *cytochrome b gene* were adapted and developed, respectively. To verify the specificity the primer systems were tested against the examined domestic poultry species as well as against beef, bison, sheep, goat, horse, kangaroo and ostrich. To prevent false positive results no-template-controls (NTC) were determined within each experiment. As an example in figure 2 the polyacrylamide gels visualised using ethidium bromide following PCR with the species specific primer system for pheasant-tissue are shown.

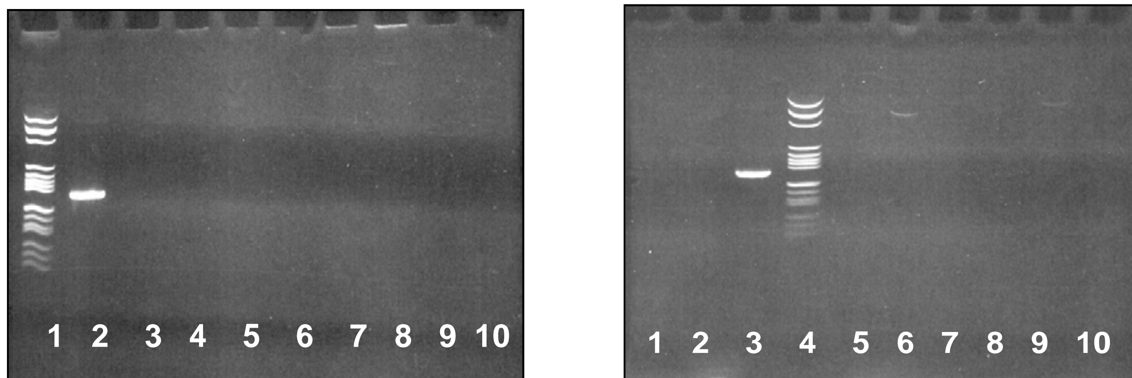

Fig. 2: Specific detection of pheasant using the *cytochrome b gene* primer system for pheasant-tissue
Right: 1: marker pBR322, 2: pheasant, 3: guinea fowl, 4: quail, 5: chicken, 6: turkey, 7: goose, 8: duck, 9: pig, 10: NTC; Left: 1: cattle, 2: bison, 3: pheasant, 4: marker pBR322, 5: sheep, 6: goat, 7: horse, 8: ostrich, 9: kangaroo, 10: NTC

Only pheasant-tissue shows a distinct amplification product. Non-specific side products do not exist. To prove the specificity of the primer systems not only in meat, but also in processed meat products a series of relevant commercially available meat-products were tested.

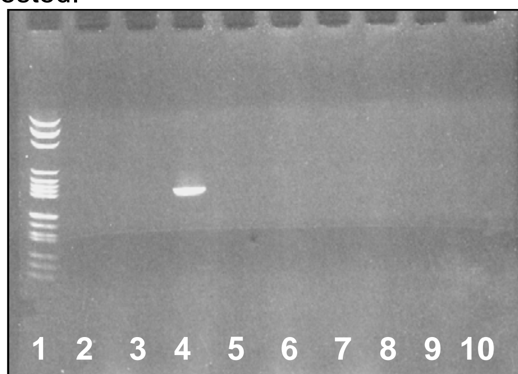

Fig. 3: Detection of guinea fowl in commercially available meat-products using the primer system for guinea fowl; 1: marker pBR322, 2: quail terrine, 3: pheasant terrine, 4: guinea fowl terrine, 5: goose á l'Orange, 6: muscovy duck savoury, 7: turkey-Gelbwurst, 8: poultry-wiener, 9: pig, 10: NTC

Exemplary in figure 3 the test results using the primer system for guinea fowl analyzing processed meat products are shown. In this case only the product containing guinea fowl tissue is showing a distinct PCR-product, too. There is no cross-similarity detectable regarding the analysed domestic poultry species.

Conclusions - Authentication of domestic poultry species in meat and meat products

Based on the mitochondrial *cytochrome b gene* a series of species specific primer systems was adapted and developed, respectively, that allow a strictly specific detection of chicken, turkey, duck, goose, pheasant, quail and guinea fowl in meat and processed meat products. Thus a tool was created to deal with possible adulteration using authentication with respect to food labelling as well as compliance with ingredient composition covering the range of all common domestic poultry species in the European market

Summary

In the area of meat and meat products there is a need for fast and reliable systems to enable traceability along the full chain to provide safe and high quality food for the consumer with respect to origin and processing. Traceability can not be considered as a request of legislation addressed to food business operators but it has to be also in their very own interest regarding food liability. It is indeed in the very interest of the European Commission. Evidence for this are plenty of European projects within the 5th and 6th research framework, ΣChain being only one of them dealing with traceability along the food chain. At the foundation of any endeavour toward food safety should be a solid understanding of the interrelation and complexity of the food chain itself. Starting from this basis analytical methods can be valuable instruments to improve food safety and avoid fraud. However, safe food is only achievable if all key issues of tracking and tracing along the chain are addressed with an equal and adequate amount of attention.

Acknowledgements

This study was supported by the European Commission, project ΣChain, Contract no.: FP6 – 518451.

References

Binke, R. (2004): Optimierung von DNA-analytischen Methoden zur Identifizierung und Quantifizierung von tierischen Bestandteilen in Fleischerzeugnissen. PhD-Thesis, University of Hamburg, Department of Chemistry.

Colombo, F., Marchisio, E., Pizzini, A. and Cantoni, C. (2002): Identification of goose species (anser anser) in Italian “Mortara” salami by DNA sequencing and a Polymerase Chain Reaction with an original primer pair. Meat Science, 61 (3), p. 291-294.

Commission Decision 2003/181/EC (2003): amending Decision 2002/657/EC as regards the setting of minimum required performance limits (MRPLs) for certain residues in food of animal origin (Text with EEA relevance) (notified under document number C(2003) 764). Official Journal of the European Union L 71, p. 17–18.

Commission Decision 2002/657/EC (2002): implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (Text with EEA relevance) (notified under document number C (2002) 3044). Official Journal of the European Union, L 221, p. 8–36.

Council Regulation (EEC) No 2377/90 (1990): laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin Official Journal of the European Union, L 224, p. 1–8

Council Directive 96/22/EC (1996): concerning the prohibition on the use in stock farming of certain substances having a hormonal or thyrostatic action and of ß-agonists, and repealing Directives 81/602/EEC, 88/146/EEC and 88/299/EEC Official Journal of the European Union, L 125, p. 3–9.

Council Directive 96/23/EC (1996): on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and

86/469/EEC and Decisions 89/187/EEC and 91/664/EEC. Official Journal of the European Union, L 125, p. 10–32.
Directive 2003/74/EC (2003): of the European Parliament and of the Council amending Council Directive 96/22/EC concerning the prohibition on the use in stock farming of certain substances having a hormonal or thyrostatic action and of beta-agonists. Official Journal of the European Union, L 28, p. 45–50.
Dooley, J. J., Paine, K. E., Garrett, S. D. and Brown, H. M. (2004): Detection of meat species using TaqMan real-time PCR assays. Meat Science, 68 (3), p. 431-438.
Posyniak, A., Zmudzki and J., Mitrowska, K., (2005): Dispersive solid-phase extraction for the determination of sulfonamides in chicken muscle by liquid chromatography. Journal of Chromatography A, 1087, p. 259-264.
Regulation (EC) No 1831/2003 (2003): of the European Parliament and of the Council on additives for use in animal nutrition. Official Journal of the European Union, L 268, p. 29–43.
Regulation (EC) No 178/2002 (2002): laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. Official Journal of the European Union, L 31, p. 1–24.
Von Bittner, G. and Windhorst, H. (2005): Geflügelmast in Deutschland. Weiße Reihe; Band 24.
Schwägele, F. (2005): Traceability from a European perspective. Meat Science, 71, p. 164-173.
ΣChain (2006): 6th Framework Programme (EC) Project. Developing a Stakeholders´ Guide on the vulnerability of food and feed chains to dangerous agents and substances. (FP6 – 518451) Available from http://www.sigmachain.eu

Keywords: Traceability; Species Authentication; PCR; Residues; Sulfonamides