Plan d'appui scientifique à la recherche prénormative dans le secteur alimentaire dans un contexte de développement durable

Stratégie intégrée d'analyse qualitative et quantitative des résidus de substances antimicrobiennes dans les denrées alimentaires

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1. EXECUTIVE SUMMARY

In the framework of the "Scientific Support Plan for a Sustainable Development Policy in the field of Standards for Food Products", we have developed two actions :

1.1 . Standardisation of hormone and veterinary drug residue analysis in animal products

(March 1997 – February 1998))

A new database was created which provides a carefully judged inventory of analytical methods available for the determination of residues of growth promoters (steroidal anabolic hormones, β-agonists and glucocorticoids) and veterinary drugs (antibiotics and growth inhibitors), which are or will be regulated by the European Union legal acts.

Other parts of the database involve informations on:

- legislation (Belgian and European),
- toxicological aspects of residues,
- statistics of controls performed by the authorities (Ministry of Agriculture and Institute of Veterinary Expertise – IEV – of the Ministry of Public Health).

This database is available on the Internet at***: http://139.165.180.63/OSTC/***

1.2 . Integrated strategy for the qualitative and quantitative analysis of residues of antimicrobial substances in food products

(from March 1998 to February 2001)

The project aimed at demonstrating the feasibility of such an integrated strategy. The strategy is based on the availability of appropriate immunoassays or other biochemical tests and of suitable chemical methodology which is considered commonly available to a well equipped control laboratory.

A pilot methodology for the identification, the confirmation and the quantitative determination of residues of commonly encountered antibacterial substances in animal tissue and products is set up. After thorough validation in agreement with the internationally accepted standards, the methodology have been applied in practice on real samples collected in slaughterhouses by IEV meat inspectors. These samples had been considered as positive for the presence of antibacterial agents after testing with the "New Belgian Kidney Test". We received also from IEV kidney and meat samples from animals recognized as negative for the presence of antibacterial substances in their kidney according to the official control. These samples were used as "blanks" to set up our methods of analysis and during their validation in our quality controls.

Presently in Belgium, the test which is applied to determine if a food sample is positive or negative for the presence of antibacterial substances, is the "Belgian Kidney test". This "pre-screening" microbiological test, which is applied on kidneys of slaughtered animals, is based on the antibacterial activity of antibiotics. It only allows to detect the presence of inhibiting substances in kidney exsudate. But it does not allow the identification of the inhibiting substances nor the quantification of their concentrations in edible parts of the animal carcass, particularly in skeletal muscles (meat). To make progress in the application of MRLs, it is thus necessary to apply simple and rapid methods which could allow the identification of antimicrobial substances. These "new" methods will thus complete the

results obtained from the kidney test in order to establish if the antibacterial substance is permitted or banned in animal products and to determine if its concentration is compatible with the MRL regulation.

An efficient system for the control of antibacterial residues should consist of four stages:

1. Pre-screening at the level of the slaughterhouses by means of a microbiological test for the presence of bacterial growth inhibiting substances. This phase is already fully operational in Belgium (Belgian kidney test).
2. Selective screening of the positives, obtained sub 1, by means of immunoassays in order to come to an identification of the group of growth inhibitors (sulphonamides, beta-lactams, tetracyclins, aminoglycosides, macrolides, (fluoro)quinolones, phenicols)
3. Chemical identification (using GC, GC/MS, HPLC/UV, LC/MS methods) within the group of the individual growth inhibitors.
4. Quantitative assay of the identified residue in view of the established Maximum Residue Limit (Council Regulation N° 2377/90).

The latter three phases are not yet operational due to the lack of an adequate analytical strategy.

The project aims at demonstrating the feasibility of such an integrated strategy. After a thorough literature search and owing to our developing expertise in the framework of *part 1* of the project, it became clear that such a system does not yet exist. The selection of the antibacterials which have to be analysed was based on the previous experience of both laboratories involved in this project in the determination of given families of antibacterial agents and the availability of appropriate immunoassays, or other biochemical tests, and of suitable physicochemical methodology, which is commonly available to a well equipped laboratory.

The antibacterials which were most often found as residues in animal products belong to one of the following seven families of substances: beta-lactams, (fluoro)quinolones, macrolides, chloramphenicol, tetracyclines, aminoglycosides and sulphonamides.

Previous experience of the services of the Ministry of Public Health in the control of residues in animal products has shown that sulphonamides, tetracyclines and beta-lactam antibiotics are the most often detected antibacterials in kidney and meat. For this reason, only samples which were found positives in the kidney test but negative for these 3 families, have been analysed for the presence of chloramphenicol, aminoglycosides and macrolides.

The objectives of the two projects were the following:

RESULTS

University of Ghent work package

The antibacterial families which were studied in Ghent involved: sulphonamides, tetracyclines and aminoglycosides.

Sulphonamides

Screening

As no multiresidue ELISA kit was available on the market for sulphonamides, High Performance Thin Layer Chromatography (HPTLC) was selected as screening method. After development in HPTLC, the spots corresponding to the residues were still visible at concentrations lower than the MRLs (100 µg/kg). The extraction yield was very high and no

interferences were noted. This technique allows the determination of 30 samples per day and per analyst. A total of 161 samples (kidney + muscle) were analysed with this method, from which 21 (13%) were found positive for the presence of sulphonamides.

Confirmation by LC-MS/MS
A simple extraction procedure was adopted. After optimalization of the chromatography as well as the mass spectrometry conditions, the method was validate taking into account the following parameters: linearity, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ) for kidney as well as for meat samples. It appeared that that this method is in agreement with the quality criteria defined in the European Union for analysing residues in food.
From the 42 samples (kidney and muscle) found positive in the screening phase, 28 (67%) were confirmed by GC-MS/MS without ambiguity. This corresponds to about 1/3 of the samples considered as positive after the screening but which were found in reality false positive when MRL values are taken in consideration.

Tetracyclines

Screening
The applicability of the commercial Ridascreen tetracycline kit (R-Biopharm) was tested. A total of 104 meat and 99 kidney samples, which had been found positive in the Belgian Kidney test, were analysed. For the meat samples, 28 (27%) appeared to contain more than 100ppb tetracyclines (MRL value). For the kidney samples, 13 were considered as positive at this stage. This represents a total of 41 positive samples.

Confirmation by LC-MS/MS
A simple extraction procedure was adopted. The analyses were performed on the same apparatus as used for sulphonamides. After optimalization , the method was validated using the same criteria as for sulphonamides.
From the 41 samples (kidney and muscle) found positive in the screening phase, 20 were confirmed by GC-MS/MS without ambiguity. This corresponds to about half of the samples considered as positive after the screening but which were found in reality false positive when MRL values are taken in consideration.

Aminoglycosides
Screening
Neomycin was selected as representative of this large group of antibiotics. Actually, it is a mixture of 3 different forms: neomycine A,B and C. Several assay kits are available. The EIA kit from Euro-Diagnostica was finally selected after a short comparison study.
Large variations were observed in the results and it was not possible to overcome this problem within the time allowed by this research. The difficulties are probably due to unreproducibility of the extraction yields.
From the remaining samples that had not been found positives for sulphonamides nor tetracyclins, namely 65 kidneys and 66 meat samples, 7 (11%) kidneys and 5 (8%) meat samples were tested as positive in screening. Due to the lack of precision of the results, it was not possible to compare to the neomycin MRL value.

Confirmation by LC-MS/MS
Due to the difficulties encountered with the yield of extraction, it was considered that the otpimization of the LC-MS/MS method of analysis was not possible at this stage.

University of Liège work package

The antibacterial families which were studied in Liège involved: beta-lactam antibiotics, macrolides and chloramphenicol. During the course of this contract, (fluoro)quinolones were also considered taking into account their particular importance in the problem of the increase of antibacterial resistance to antibiotics.

Beta-lactam antibiotics

Screening

We have adapted, validated and use, for analysing this type of residues in kidney and meat, a new test on strip, Beta-STAR (UCB Bioproducts), marketed for the antibiotic monitoring in milk. For animal tissue analysis, the strip test was preceded with a solid phase extraction (hydroxyapatite columns) of contaminating proteins in the aqueous extract. In these conditions, penicilline G, ampicilline and amoxicilline are detected at concentrations close to their MRLs. For oxacilline and cloxacilline, they are detected concentrations lower than LMR/2.

Confirmation / quantification

High performance liquid chromatography (HPLC) was selected as the analytical method for confirming the presence of beta-lactams in kidney or meat samples and for their quantitation. It was observed that it is mandatory to adopt special working conditions to avoid degradation of these substances during the analytical process. LOD was evaluated to 15 and 75 µg/kg according to the type of compound. LOQ is 25 µg/kg for penicilline G, amoxicilline and ampicilline and 150 µg/kg for oxacilline and cloxacilline. This method of analysis is thus well suited for penicilline G, amoxicilline and ampicilline (MRL = 50 µg/kg) and for oxacilline and cloxacilline (MRL = 300 µg/kg).

From the remaining samples that had not been found positives for other antibacterials, namely 17 kidney samples examined, only one was positive in screening and confirmed for ampicilline at 1553 µg/kg.

Macrolides

Screening

The method used for the detection of macrolide residues was a radio-receptor assay developed and validated in our laboratory. This technique was applied for analysing kidney and meat samples collected by IEV meat inspectors.

Confirmation / quantification

A LC-MS/MS method was developed and partly validated.

From the 17 kidney samples examined, only one was positive in screening and the presence of spiramycine was confirmed at 93.5 µg/kg.

Chloramphenicol

Screening

The ELISA Ridascreen was used for the determination of chloramphenicol (a banned substance in veterinary medicine). Other antibacterial compounds of this family, such as thiamphenicol, were not taken in consideration due to the lack of multiresidue test kits available on the market.

Confirmation / quantification
A LC-MS/MS method was developed for the quantitative analysis of chloramphenicol.

From the 17 kidney samples examined, 4 were positive positive and their concentrations were estimated to 0.4, 0.6 (2) and 1.2 µg/kg.

(Fluoro)quinolones
Screening
Screening test is not commercially available. A new biochemical test is still in development at the University of Liège.
For this reason, the samples collected by IEV were not examined for the presence of this type or residues.
Confirmation / quantification
The conditions of extraction and purification of these residues from kidney and meat samples have been studied for the main substances of this family of antibacterials and a LC-MS/MS method of analysis was set up and validated. It was found applicable as a multiresidue method. Quantitation will soon be optimised by using a new deuterated standard, this will allow a complete validation of the method.

Our project had to lead to:
1° a strengthening and an improvement of the link between the scientific potential of research centres (Universities of Ghent and Liège) and the regulatory authorities concerned by the standardisation in the food product sector (within the future Federal Agency for the Safety of the Food Chain) and also with the OIVO/CRIOC (a Belgian Centre of Research and Information of Consumer Organizations). The OIVO/CRIOC will be informed about the results of the project and coached in the translation towards the consumers.
2° an acceleration and a better coordination of the standardization process in a context of sustainable development in the sector of animal productions.

CONCLUSIONS AND RECOMMANDATIONS

The important changes in the strategy of control of residues in foodstuffs and in food products required in the European Directives (EC/96/23) and Council Regulation EEC N°2377/90 that have been implemented rather recently require also a modification of the strategy of the use in the laboratories of analytical methods for the determination of veterinary drug residues and particularly of antibiotics. For these last compounds, the question is asked about the demonstration of the presence of substances inhibiting bacterial growth that could no longer be sufficient to reject the animal carcass: after identification of the inhibiting substance, its concentration in animal products edible for human consumers would have to be determined and compared to the Maximum Residue Limits (MRLs) in the corresponding foodstuffs. Reliable and reproducible quantitative methods of analysis are thus needed. This will be possible only after complete validation and standardisation of analytical methods.

Our project has largely reached its objectives:

- an inventory of the control tools consisting of commercially available methods for the post-screening of antibiotic residues was established. Some of them have been applied to the determination of antibiotic residues in animal kidneys. Their complete validation and standardisation are in progress;

- published physico-chemical methods for the confirmatory analysis and quantitation of antibiotic residues have been applied to kidney samples collected in Belgian slaughterhouses. Those which were found satisfactory were partly validated and are on the verge to be standardised in view of their inter-laboratory evaluation;
- new analytical approaches were followed when needed after a negative evaluation of the performances of existing methods of analysis;
- progress have been done is the development of laboratory networks at the national and European levels in the field of the control of antibiotic residues in foodstuffs of animal origin.

Our project was clearly in line with the general objectives of the *Scientific Support Plan for a Sustainable Development Policy* in the food product sector for the harmonization of methods of analysis. This should allow a better protection of the consumer health concerning potentially harmful residues of certain veterinary drugs such as antibiotics.
The validation of the analytical methods developed and tested in our project will have still to be done in close collaboration with other laboratories involved in the official control of veterinary drug residues in foodstuffs of animal origin. This will contribute to the integration of results and will allow identification of domains in the food production chain for which an effort of standardization should be needed in the frame work of sustainable development. The project will contribute to the creation of an inventory of European and worldwide initiatives and to the development of reliable data bank largely available. It will examine actions taken at the Belgian and international level in the field of food product standards (more precisely, methods of residue analysis in food products) in a context of sustainable development and it will allow the definition of the Belgian contribution at the international level (especially the European level).

Validation of analytical methods developed and tested in our project will still have to be completed in collaboration with other laboratories involved in the official control fo residues. This will allow the identification of domains in the sector of the chain of food production in which a special standardisation effort is still necessary in the framework of sustainable development. The project will have to contribute in the setting up of an inventory of the initiatives at European and worldwide levels et in the development of databank easily available. It will examine the actions conducted in Belgium and at the international level in the domain of standardisation of food products (more specifically concerning the analytical methods of residues in foodstuffs of animal origin).