ANNEX II

GENERAL REQUIREMENTS TO BE SATISFIED BY THE DOSSIER PROVIDED FOR IN ARTICLE 3

3. SECTION III: STUDIES CONCERNING SAFETY OF THE ADDITIVE

3.2. Studies concerning the safety of use of the additive for consumers

The aim is to evaluate the safety of the additive for the consumer and to establish potential residues of the additive or its metabolites in food derived from animals given feed or water containing or treated with the additive.

3.2.1. Metabolic and residue studies

The establishment of the metabolic fate of the additive in the target species is a determinant step in the identification and quantification of the residues in the edible tissues or products derived from the animals given the feed or water containing the additive. Studies must be submitted concerning the absorption, distribution, metabolism and excretion of the substance (and its metabolites).

Studies must be carried out using internationally validated test methods and shall be performed in accordance with European legislation in force or OECD Guidelines for methodological details and according to the principles of GLP. The study shall respect the rules on animal welfare laid down by European Community legislation, and they shall not be repeated if not necessary.

Metabolic and residue studies on the target animal(s) shall be performed with the active substance incorporated in the feed (not given by gavage unless it is properly justified).

Structural identification of metabolites representing more than 10 % of the total residues in the edible tissues and products and more than 20 % of the total residues in the excreta shall be established. If the metabolic pathway of the active substance raises any toxicological concerns, metabolites below the above limits shall be identified.

Kinetic studies of the residues will form the basis for the calculation of consumer exposure and the establishment of a withdrawal period and MRLs, if necessary. A proposal for a marker residue shall be provided.

For some additives, depending on their nature or use, it may not always be necessary to carry out metabolic and residues studies.

3.2.1.1. Metabolic studies

The purpose of metabolic studies is to evaluate the absorption, distribution, biotransformation and excretion of the additive in the target species.

The studies required are:

(1) metabolic balance following a single dose administration of the active substance at the doses proposed for use (total amount corresponding to the daily intake) and possibly a multiple dose (if justified) to assess an approximate rate and extent of the absorption, distribution (plasma/blood) and excretion (urine, bile, faeces, milk or eggs, expired air, excretion via gills) in male and female animals, where appropriate; and

(2) metabolic profiling, identification of the metabolite(s) in excreta and tissues and distribution in tissues and products shall be established following repeated dose administration of the labelled compound to animals to the steady state (metabolic

equilibrium) identified by plasma levels. The dose applied shall correspond to the highest dose proposed for use, and shall be incorporated into the feed.

3.2.1.2. Residue studies

Consideration shall be given to the amount and the nature of non-extractable residues in edible tissues or products.

Residue studies are required for all substances for which metabolic studies are needed.

If the substance is a natural constituent of body fluids or tissues or is naturally present in significant amounts in food or feedingstuffs, the requirement for residue studies is limited to the comparison of the tissue/product levels in an untreated group and in the group supplemented with the highest dose claimed.

For major species, studies shall simultaneously evaluate the total residues of toxicological significance and identify the marker residue of the active substance in edible tissue (liver, kidney, muscle, skin, skin + fat) and products (milk, eggs and honey). The marker residue is the residue selected for assay whose concentration has a known relationship to the total residue of toxicological concern in the tissues. Studies shall also show the permanence of the residues in the tissues or products to establish an appropriate withdrawal period.

For the determination of a withdrawal period, the suggested minimum number of animals sampled and/or products at each time point are the following:

— edible tissues:
  — bovines, sheep, pigs and minor species 4;
  — poultry 6;
  — salmonids and other fish 10.
— products:
  — milk 8 samples per time point;
  — eggs 10 eggs per time point;
  — honey 8 samples per time point.

Appropriate sex distribution shall be considered.

The residues shall be measured at zero withdrawal time (steady state) and at least three other time sampling points.

A proposal for a marker residue shall be provided.

Studies on the absorption, distribution and excretion, including the identification of main metabolites must be performed in the laboratory animal species in which the lowest NOAEL was obtained, or by default in the rat (both sexes). Additional studies on particular metabolites may be necessary if these metabolites are produced by target species and are not formed to a significant extent in the laboratory species.

3.2.1.3. Metabolic and disposition studies

A metabolism study including the metabolic balance, metabolic profile and identification of the main metabolites in the urine and faeces shall be performed. If another laboratory species shows a marked difference in the sensitivity from the rat, additional information will be required.

3.2.1.4. Bioavailability of residues

The assessment of the risks for the consumers related to bound residues in animal products may take into account an additional safety factor on the determination of their bioavailability using appropriate laboratory animals and recognised methods.

3.2.2. Toxicological studies

The safety of the additive is assessed on the basis of the toxicological studies performed *in vitro* and *in vivo* on laboratory animals. They generally include measurements of:

(1) acute toxicity;

(2) genotoxicity (mutagenicity, clastogenicity);

(3) sub-chronic oral toxicity;

(4) chronic oral toxicity/carcinogenicity;

(5) reproduction toxicity including teratogenicity; and

(6) other studies.

Further studies providing additional information necessary for the assessment of the safety of the active substance and its residues shall be conducted if there is any reason for concern.

On the basis of the results of these studies a toxicological NOAEL must be established.

Additional studies on particular metabolites may be necessary if these metabolites are produced by target species and are not formed to a significant extent in the laboratory test species. If metabolic studies are available in humans, data shall be taken into consideration in deciding the nature of eventual additional studies.

Toxicological studies must be carried out with the active substance. If the active substance is present in a fermentation product, the fermentation product shall be tested. The fermentation product tested must be identical to that to be used in the commercial product.

Studies must be carried out using internationally validated test methods and shall be performed in accordance with European legislation in force or OECD Guidelines for methodological details and according to the principles of GLP. The studies involving laboratory animals shall respect the rules on animal welfare laid down by European legislation and they shall not be repeated if not necessary.

3.2.2.1. Acute toxicity

Acute toxicity studies are required to classify and to provide limited characterisation of the toxicity of the compound.

Acute toxicity studies shall be carried out in at least two mammalian species. One laboratory species may be replaced by a target species, if appropriate.

It will be not necessary to determine a precise LD_{50}; an approximate determination of the minimum lethal dose is considered sufficient. The maximum dosage shall not exceed 2 000 mg/kg body weight.

In order to reduce the number and the suffering of the animals involved, new protocols for acute dose toxicity testing are continually being developed. Studies carried out by these new procedures will be accepted, when properly validated.

OECD Guidelines 402 (acute dermal toxicity), 420 (Fixed Dose Method), 423 (Acute Toxic Class Method) and 425 (Up-and-Down Procedure) should be followed.

3.2.2.2. Genotoxicity studies including mutagenicity

To identify active substances and, if appropriate, their metabolites and degradation products with mutagenic and genotoxic properties, a selected combination of different genotoxicity tests must be carried out. If appropriate the tests shall be performed without and with mammalian metabolic activation and the compatibility of the test material with the test system shall be taken into account.

The core set comprises the following tests:

(1) induction of gene mutations in bacteria and/or in mammalian cells (preferably the mouse lymphoma tk assay);

(2) induction of chromosomal aberrations in mammalian cells; and

(3) *in vivo* test in mammalian species.

Additional tests may be needed depending on the outcome of the above mentioned tests and taking into consideration the whole toxicity profile of the substance, as well as its intended use.

Protocols should be in line with OECD Guideline 471 (*Salmonella typhimurium* Reverse Mutation Test), 472 (*Escherichia coli* Reverse Mutation Test), 473 (*in vitro* Mammalian Chromosomal Aberration Test), 474 (Mammalian Erythrocyte Micronucleus Test), 475 (Mammalian Bone Marrow Chromosomal Aberration Test), 476 (*in vitro* Mammalian Cell Gene Mutation Test) or 482 (Unscheduled DNA Synthesis in Mammalian Cells *in vitro*), as well as other relevant OECD Guidelines for *in vitro* and *in vivo* assays.

3.2.2.3. Sub-chronic repeated dose oral toxicity studies

To investigate the sub-chronic toxic potential of the active substance, at least one study on a rodent species must be submitted with duration of at least 90 days. If deemed necessary, a second study must be performed with a non-rodent species. The test item must be administered orally with at least three levels in addition to a control group to obtain a dose response. The maximum dose used should normally be expected to reveal evidence of adverse effects. The lowest dose level should not be expected to produce any evidence of toxicity.

Protocols for these studies should be in line with the OECD Guidelines 408 (rodents) or 409 (non-rodents).

3.2.2.4. Chronic oral toxicity studies (including carcinogenicity studies)

To investigate the chronic toxic potential and carcinogenic potential, a chronic oral toxicity study must be carried out in at least one species, and shall be of at least 12 months' duration. The species chosen shall be the most appropriate on the basis of all available scientific data, including the results of the 90-day studies. The default species is the rat. If a second study is requested, a rodent or a non-rodent mammalian species shall be used. The test item must be administered orally with at least three levels in addition to a control group to obtain a dose response.

If the chronic toxicity study is combined with an examination of carcinogenicity, then the duration shall be extended to 18 months for mice and hamsters, and to 24 months for rats.

Carcinogenicity studies may not be necessary if the active substance and its metabolites:

(1) give consistently negative results in the genotoxicity tests;

(2) are not structurally related to known carcinogens; and

(3) give no effects indicative of potential (pre)neoplasia in chronic toxicity assays.

Protocols should be in line with OECD Guideline 452 (chronic toxicity study) or 453 (combined chronic toxicity/carcinogenicity study).

3.2.2.5. Reproduction toxicity studies (including prenatal developmental toxicity)

To identify possible impairment of male or female reproductive function or harmful effects on progeny resulting from the administration of the active substance, studies of reproductive function must be carried out by:

(1) two generation reproduction toxicity study; and

(2) prenatal developmental toxicity study (teratogenicity study).

For new trials validated alternative methods reducing the use of animals can be used.

3.2.2.5.1. Two generation reproduction toxicity study

Studies of reproductive function must be carried out and extend over at least two filial generations (F1, F2) in at least one species, usually a rodent, and may be combined with a teratogenicity study. The substance under investigation shall be administered orally to males and females at an appropriate time prior to mating. Administration shall continue until the weaning of the F2 generation.

All relevant fertility, gestation, parturition, maternal behaviour, suckling, growth and development of the F1 offspring from fertilisation to maturity and the development of the F2 offspring to weaning must be carefully observed and reported. Protocols for the reproduction toxicity study should be in line with OECD Guideline 416.

3.2.2.5.2. Prenatal developmental toxicity study (teratogenicity study)

The objective is to detect any adverse effects on the pregnant female and the development of the embryo and foetus as a result of exposure from implantation through the entire gestation period. Such effects include enhanced toxicity in the pregnant females, embryo-foetal death, altered foetal growth and structural abnormalities and anomalies in the foetus.

The rat is usually the species of choice for the first study. If a negative or an equivocal result for teratogenicity is observed, another developmental toxicity study shall be conducted in a second species, preferably the rabbit. If the rat study is positive for teratogenicity, a study in a second species is not necessary except where a review of all the core studies indicates that the ADI would be based on the rat teratogenicity. In this case a study in a second species would be required to determine the most sensitive species for this endpoint. Protocols should be in line with OECD Guideline 414.

3.2.2.6. Other specific toxicological and pharmacological studies

Further studies providing additional information useful for the assessment of the safety of the active substance and its residues shall be conducted if there are reasons for concern. Such studies may include examination of pharmacological effects, effects in juvenile (prepubertal) animals, immunotoxicity or neurotoxicity.

3.2.2.7. Determination of No Observed Adverse Effect Levels (NOAEL)

The NOAEL is generally based on toxicological effects, but pharmacological effects might occasionally be more appropriate.

The lowest NOAEL shall be selected. All findings from previous sections together with all other relevant published data (including any relevant information on the effects of the active substance on human) and information, where appropriate, on chemicals having a closely related chemical

structure shall be taken into consideration in identifying the lowest NOAEL, expressed as mg per kg body weight per day.

3.2.3. Assessment of consumer safety

Consumer safety is assessed by a comparison of the established ADI (Acceptable Daily Intake) and calculated theoretical intake of the additive or its metabolites from food. In the case of vitamins and trace elements, UL (Tolerable Upper Intake Level) can be used in place of ADI.

3.2.3.1. Proposal of the acceptable daily intake (ADI) for the active substance(s)

The acceptable daily intake (ADI) (expressed as mg of additive or additive related material per person per day) is derived by dividing the lowest NOAEL (mg per kg body weight) by an appropriate safety factor and multiplying by the average human body weight of 60 kg.

An ADI shall, where appropriate, be proposed. An ADI can also be 'not specified' because of low toxicity in animal tests. An ADI shall not be proposed if the substance shows genotoxic or carcinogenic properties relevant to humans.

The setting of an ADI normally requires the similarity of metabolic fate of the active substance in the target animals and laboratory animals (see 3.2.1.4 Bioavailability of residues) which ensures that consumers are exposed to the same residues as the laboratory animals used in toxicological studies. If not, additional studies in a second laboratory animal species or with the metabolites specific to the target species may still allow an ADI to be set.

The safety factor used to determine the ADI for a particular additive will take into consideration the nature of the biological effects and the quality of the data used to identify the NOAEL, the relevance of these effects to man and their reversibility and any knowledge of the direct effect(s) of the residues in human.

A safety factor of at least 100 in calculating the ADI (if a full toxicological package has been provided) shall be employed. Where data on the active substance are available for human, a lower safety factor may be acceptable. Higher safety factors might be applied to account for additional sources of uncertainty in data or where the NOAEL is set on the basis of a particular critical endpoint, such as teratogenicity.

3.2.3.2. Tolerable upper intake level (UL)

For some additives it may be more appropriate to base the safety assessment on the UL, which is the maximum level of total chronic daily intake of a nutrient (from all sources) judged (by national or international scientific bodies) to be unlikely to pose a risk of adverse health effects to consumers or to specific groups of consumers.

The dossier shall contain data to demonstrate that use of the additive would not lead to a situation in which the UL could be exceeded considering all possible sources of the nutrient.

If the resulting residue levels of the nutritional additive or its metabolite(s) in products of animal origin are higher than what is considered normal or expected for these products, this shall be clearly indicated.

3.2.3.3. Consumer exposure

The total intake of the additive and/or its metabolites from all sources by the consumer shall be below the ADI or UL.

Calculation of the theoretical intake from food of animal origin shall be performed considering the concentration (total residues as the arithmetic mean and the highest single value) measured in tissues and products at the termination of use of the additive. In addition, if necessary, at

the different withdrawal times, the human daily food consumption values shall be determined following a worst case scenario.

For additives intended for multi-species, the exposure from tissues shall be independently calculated for mammals, birds and fish and the highest value taken. Where appropriate, exposure from milk and eggs shall be added to this figure. For example, where an additive is applied for lactating mammals and laying birds, the respective highest edible tissue values are added to those for milk and egg consumption. Where the additive is applied for fish and laying birds and lactating mammals, the respective highest edible tissue values are added to those for egg and milk consumption. Other combinations shall be envisaged in the same way.

In certain situations (e.g. some nutritional and sensory additives or additives intended for minor species) it may be appropriate to subsequently refine the human exposure assessment using more realistic consumption figures, but still keeping the most conservative approach. Where this is possible this shall be based on Community data.

TABLE 1

Theoretical daily human consumption figures (g tissues or products)

	Mammals	**Birds**	**Fish**	**Other**
Muscle	300	300	300[a]	
Liver	100	100	—	
Kidney	50	10	—	
Fat	50[b]	90[c]	—	
+ Milk	1 500	—	—	
+ Eggs	—	100	—	
+ Honey				20

a Muscle and skin in natural proportion.

b For pig 50 g of fat and skin in natural proportion.

c fat and skin in natural proportion.

3.2.3.4. Proposal for maximum residue limits (MRLs)

Maximum residue limit means the maximum concentration of residues (expressed as µg marker residue per kg of edible wet tissue or product) which may be accepted by the Community to be legally permitted or recognised as acceptable in food. It is based on the type and amount of residue considered to be without any toxicological hazard for human health as expressed by the ADI. An MRL cannot be set in the absence of an ADI.

When establishing MRLs for feed additives, consideration is also given to residues that come from other sources (e.g., food of plant origin). Furthermore, the MRL may be reduced to be consistent with the conditions of use of feed additives and to the extent that practical analytical methods are available.

Where appropriate, individual MRLs (expressed as mg marker residue per kg of edible natural tissue or product) shall be set for different tissues or products of the target animal species. The individual MRLs in different tissues or products shall reflect the depletion kinetics and the variability of the residue levels within those tissues/products in the animal species intended for use. Variability shall normally be reflected by using the 95 % confidence limit of the mean. If the

confidence limit cannot be calculated due to a low number of samples, variability is expressed by taking the highest individual value instead.

Studies concerning the Maximum Residue Limits of coccidiostats and histomonostats must be carried out following the appropriate rules in force for veterinary medicinal products (Volume 8 'The rules governing medicinal products in European Union — Notice to applicants and guidelines. Veterinary medicinal products. Establishment of maximum residue limits (MRLs) for residues of veterinary medicinal products in foodstuffs of animal origin'. October 2005).

The studies to establish maximum residue limits for additive categories other than coccidiostats and histomonostats, where necessary, shall be provided according to this Annex.

To determine the consumer exposure to the total residues (as calculated under 3.2.3.3.), the proposed MRLs for the different tissues or products shall take into account the ratio of marker residue to total residue (Table 2).

TABLE 2

Definitions used in deriving an MRL

$_{i-j}$	Individual tissues/products (liver, kidney, muscle, skin + fat, milk, eggs, honey) at different times
MRL_{i-j}	Maximum residue limit in tissues/products (mg marker substance kg^{-1})
Qt_{i-j}	Daily human consumption of individual tissues/products (kg) set by Table 1 or its refinement
TRC_{i-j}	Total residue concentration in individual tissues/products (mg kg^{-1})
MRC_{i-j}	Marker residue concentration in individual tissues/products (mg kg^{-1})
$RMTR_{i-j}$	Ratio MRC_{i-j} to TRC_{i-j} for individual tissues/products
$DITR_{i-j}$	Dietary intake for individual tissues/products calculated from total residues (mg) $DITR_{i-j} = Qt_{i-j} \times TRC_{i-j}$
$DITR_{MRLi-j}$	Dietary intake calculated from MRLs (mg) of individual tissues/products $DITR_{MRLi-j} = Qt_{i-j} \times MRL_{i-j} \times RMTR_{i-j}^{-1}$

The measured values for TRC and MRC shall be inserted as appropriate in the template shown in Table 3, and the other values calculated. Where a full data set is not available because values fall below the limit of detection (LOD), an extrapolation of RMTR may be acceptable.

Deriving an MRL can only be performed if the sum of the individual DITRs is below the ADI. If the ADI is exceeded, an alternative would be to use data from a longer withdrawal time or lower dosages. A first proposal for an MRL can be obtained using the MRC value as a guide and taking into consideration the LOQ of the analytical method. The sum of the $DITR_{MRL}$ obtained

from the proposed MRLs must be below the ADI and close to the sum of the individual DITRs. If the ADI is exceeded, then a lower MRL shall be proposed and the comparison repeated.

For certain additives, residues could arise below the MRL values in milk, eggs or meat which could nonetheless interfere with food quality in particular food processing procedures. For such additives, it may be appropriate to consider a 'maximum (food product) processing compatible residue' (MPCR) in addition to establishing MRL values.

TABLE 3

Template for deriving a MRL proposal

	Liver	**Kidney**	**Muscle**	**Skin + fat**	**Milk**	**Eggs**	**Honey**	**Sum**
TRC[a] (mg kg^{-1})								——
MRC[b] (mg kg^{-1})								——
RMTR[b]								——
DITR[c] (mg)								
MRL proposed (mg kg^{-1})								——
$DITR_{MRL}$ (mg)								

a Considering the proposed withdrawal time.

b Ideally established at the same time as TRC.

c Calculated from TRC values.

3.2.3.5. Proposal for a withdrawal period

The withdrawal time comprises the period after cessation of the administration of the additive which is necessary to enable the residue levels to fall below the MRLs.