



GUIDELINES FOR THE ASSESSMENT OF ADDITIVES IN FEEDINGSTUFFS

PART II: ENZYMES AND MICRO-ORGANISMS

GENERAL ASPECTS

This document is intended as a guideline for establishing dossiers on preparations being submitted for authorisation as additives in feedingstuffs or a new usage of an authorised additive. The term "additive", as used in these guidelines refers to the active substance(s) or agent(s) or to the preparations containing the active substance(s) or agent(s) in the state in which they will be incorporated in premixtures and feedingstuffs. Part I of these guidelines, adopted on 22 October 1999¹, dealt with "chemically specified substances" and included all additives not being enzymes or micro-organisms. Part II deals specifically with those additives in which the active agents are enzymes or viable micro-organisms.

The dossiers must enable an assessment to be made of the additives based on the present state of knowledge and make it possible to ensure their compliance with the fundamental principles laid down for their admission, which are the subject of the provisions of Article 3A of Council Directive 70/524/EEC². The dossiers should include detailed reports of the studies done, presented in the order and with the numbering proposed in these guidelines. They should include references and copies of all published scientific data relevant to the evaluation of the additive. An electronic version of the dossier should be made available for evaluation purposes. Each dossier shall contain an adequate summary.

The studies are intended to ensure the safety of use of the additive: a) to the target species at the proposed levels of incorporation in the feedingstuff; b) to those likely to be exposed to the additive by respiratory, other mucosal, eye or cutaneous contact while handling the additive itself or when incorporated into premixtures or feedingstuffs; c) to consumers who ingest food products from animals given the additive, d) to the animals and the human-beings through the selection and spread of antimicrobial resistance genes; e) to the environment arising from the additive itself or by products derived from the additive and excreted by animals. As a general rule, studies to establish the identity, conditions of use, physico-chemical properties, methods of determination, efficacy of the additive, and effects on target species must be provided. When the additive is intended

¹ Report of the Scientific Committee on Animal Nutrition on the revision of the guidelines for the assessment of additives in animal nutrition, adopted on 22 October 1999.

² OJ EC N° L 270 of 14.12.1970, p. 1.

for a category of animals belonging to a defined species, efficacy studies must be performed on this target category.

The studies necessary for the evaluation of risks to human health or the environment will depend essentially on the nature of the additive and the circumstances of its use. In this respect, no strict rule is applicable. However, it will be assumed that all enzymes and micro-organisms are respiratory sensitisers (R42) unless convincing evidence to the contrary is provided. Consequently, attention will be paid to the physical nature of the formulation, which should minimise this risk to workers handling the product. In other respects, enzymes *per se* are not expected to be harmful to the target species, consumers of products from animals fed enzyme-treated feed or to the wider environment. Micro-organisms should be selected from taxonomic groups not normally able to induce clinical symptoms in healthy humans or animals and should be demonstrated unable to produce any toxins or virulence factors associated with related organisms. Acquired resistance genes to antibiotics of human clinical or veterinary importance also should not be present. Micro-organisms meeting these criteria similarly are not expected to be harmful to the target species, consumers of products from animal fed microbial products or to the wider environment. To confirm the absence of deleterious hazards to consumers from fermentation co-products or unexpected contaminants, oral toxicity studies and genotoxicity tests are required for each fermentation product incorporated into the final preparation.

Enzymes from a genetically modified source or genetically modified micro-organisms submitted for assessment shall have been subject to an evaluation to check compliance with the requirements of Council and Parliament Directive 2001/18/EC³ and Council Directive 90/219/EEC⁴ and will subsequently, for the purposes of these guidelines, be treated as any other additive.

Where possible, studies should be done and reported according to appropriate quality standards (*e.g.* Good Laboratory Practice pursuant to Council Directive 87/18/EEC of 18 December 1986, on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances)⁵. EU or OECD methodological guidelines may be referred to for advice on how to conduct studies.

Large animal studies should, at least, meet the quality standards laid down in these guidelines.

Study of the metabolic fate of the additive is not required unless there is evidence of the toxicity of a co-product of the fermentation.

Reasons must be given for the omission from the dossier of any data prescribed in these guidelines.

³ OJ EC N° L 106 of 17.04.2001, p. 1.

⁴ OJ EC N° L 117 of 08.05.1990, p. 1.

⁵ OJ EC N° L 15, 17.01.1987, p. 29.

CONTENTS

1. SECTION I - Summary of the data in the dossier.....	5
2. SECTION II - Identity, characterisation and conditions of use of the additive - Methods of control.....	5
2.1. Identity of the additive	5
2.1.1. Proposed proprietary name(s)	5
2.1.2. Type of additive according to its main function	5
2.1.3. Qualitative and quantitative composition	5
2.1.4. Qualitative and quantitative composition of any impurities	5
2.1.5. Physical state of each form of the product	5
2.1.6. Manufacturing process.....	6
2.2. Characterisation of the active agents(s)	6
2.2.1. Nomenclature	6
2.2.2. Biological origin	6
2.2.3. Genetic modification.....	6
2.2.4. Compliance with the release Directive for GMOs.....	7
2.2.5. Toxins and virulence factors.....	7
2.2.6. Antibiotic production and antibiotic resistance	8
2.2.7. Other relevant properties.....	8
2.3. Characterisation of the additive: physico-chemical and technological properties	9
2.3.1. Stability of the additive	9
2.3.2. Other physico-chemical or biological properties	9
2.3.3. Incompatibilities with other feed ingredients.....	9
2.4. Conditions of use of the additive	9
2.4.1. Technological additives	9
2.4.2. Zootechnical additives	9
2.4.3. Safety Data Sheet	10
2.5. Control methods.....	10
2.5.1. General methods	10
2.5.2. Description of the qualitative and quantitative methods for routine control of the active agent in premixtures and feedingstuffs.....	10
3. SECTION III - Studies concerning the efficacy of the additive	11
3.1. Technological use	11
3.2. Studies of the effects on animals	11
3.2.1. Evidence of efficacy.....	11
3.2.2. Digestion/balance studies.....	13
3.2.3. Experimental conditions	13
3.2.4. Efficacy of multicomponent additives	14
3.3. Studies on the quality of animal produce.....	15
3.4. Studies on the effects on the characteristics of animal wastes.....	15

4.	SECTION IV - Studies concerning the safety of use of the additive	16
4.1.	Studies on target species	16
4.1.1.	Tolerance tests on target species/animal categories.....	16
4.1.2.	Effects on the microflora of the digestive tract.....	17
4.1.3.	Metabolism and residue studies	17
4.2.	Consumer safety assessment.....	17
4.2.1.	Genotoxicity studies including mutagenicity.....	18
4.2.2.	Oral toxicity studies	18
4.3.	Worker safety assessment	18
4.3.1.	Irritancy	18
4.3.2.	Skin sensitisation	19
4.3.3.	Toxic effects on the respiratory system	19
4.3.4.	Systemic toxicity.....	19
4.3.5.	Control measures	19
4.4.	Environmental risk assessment.....	19
4.4.1.	Enzyme additives	19
4.4.2.	Microbial additives	20
4.4.3.	Genetically modified micro-organisms.....	20
5.	SECTION V - Form of monograph.....	21
5.1.	Identity of the additive	21
5.2.	Specifications concerning the active agent(s).....	21
5.3.	Physico-chemical, technological and biological properties of the additive.....	22
5.4.	Control methods.....	23
5.5.	Biological properties of the additive.....	23
5.6.	Other characteristics suitable for identification of the additive	23
6.	SECTION VI - Form of identification note.....	24

1. SECTION I - SUMMARY OF THE DATA IN THE DOSSIER

The summary must follow the order of the guidelines and address all the different parts with reference to the relevant pages of the dossier. It should contain a proposal covering all the conditions for the authorisation sought.

2. SECTION II - IDENTITY, CHARACTERISATION AND CONDITIONS OF USE OF THE ADDITIVE - METHODS OF CONTROL

2.1. Identity of the additive

2.1.1. *Proposed proprietary name(s)*

2.1.2. *Type of additive according to its main function*

The intended function of the additive should be described. When possible, evidence of mode(s) of action should be included.

2.1.3. *Qualitative and quantitative composition*

The active agent(s) and all other ingredients of the additive should be listed, giving the proportion by weight of the non-active ingredients in the final product. The purity of the other ingredients should be given (e.g. pharmaceutical grade) and the extent of batch to batch variation determined. When the active component is a mixture of micro-organisms, each strain must be described separately and the numbers of each strain in the final product given. Numbers of viable micro-organisms should be given as colony forming units (c.f.u.) per unit weight. For enzymes, each declared activity should be described and the number of units of each activity in the final product given.

2.1.4. *Qualitative and quantitative composition of any impurities*

Microbial and enzyme preparations should ideally be free of contaminating micro-organisms, heavy metals and toxins from any source (e.g. mycotoxins). Where this is unavoidable they should occur at concentrations below those that give cause for concern. The protocol used for the routine screening of production batches for contaminants and impurities should be described.

2.1.5. *Physical state of each form of the product*

For solid preparations, data on particle size distribution, dusting potential and the use of processes such as encapsulation which affect the physical properties should be provided. For liquid preparations, viscosity and bulk density values are sufficient.

2.1.6. Manufacturing process

A flow chart showing the key stages in the preparation of the additive including the point(s) of introduction of the active substances and other components and any subsequent process steps affecting the blended mixture should be included.

2.2. Characterisation of the active agents(s)

2.2.1. Nomenclature

The name and taxonomic status of each micro-organism according to the latest published information in the international Codes of Nomenclature should be provided. Commonly used and accepted synonyms may be introduced in the context of the above, but in-house designations and other descriptions that would not be accepted in the scientific literature must be avoided.

For enzyme preparations, the number and systematic name proposed by the International Union of Biochemistry (IUB) in the most recent edition of “Enzyme Nomenclature” should be given for each declared activity. For activities not yet included, a systematic name consistent with the IUB rules of nomenclature should be used. Trivial names are acceptable provided that they are used consistently throughout the Dossier, can be clearly related to the systematic name and IUB number at first mention, and are unambiguous.

2.2.2. Biological origin

The biological origin of each declared enzyme activity or micro-organism should be given. All micro-organisms, whether used as a product or as a producer strain, should be deposited in an internationally recognised culture collection (preferably in the European Union) and maintained by the culture collection for the authorised life of the additive. Evidence of deposition in the form of a certificate of deposition from the culture collection, which should specify the accession number and name under which the strain is held, must be provided. In addition, all relevant morphological, physiological, and molecular characteristics necessary to identify the strain and confirm its genetic stability should be described.

2.2.3. Genetic modification

For enzymes produced using recombinant DNA technology, the recipient organism is the production strain and should be described as for 2.2.2. In addition, the donor organism should be identified and described, with particular reference to any characteristics likely to cause concern. For viable micro-organisms intended as the active agent, the nature and purpose of the genetic modification should be specified and the source of any transferred DNA identified.

2.2.4. Compliance with the release Directive for GMOs

If the additive contains or consists of a GM micro-organism within the meaning of Article 2 of Council Directive 2001/18/EC⁶ the following information must be provided:

A copy of any written consent or consents of the competent authorities to the deliberate release of the genetically modified organism(s) pursuant to Article 6 of Directive 2001/18/EC and the summary of the notification as referred in Article 11 of Directive 2001/18/EC according to the model established in Council Decision 91/596/EEC⁷;

The conditions for the placing on the market of the additive, including specific conditions of use and handling and a proposal for labelling and packaging which should comprise at least the requirements laid down in Annex IV of Directive 2001/18/EC.

For GM micro-organisms used as source of enzymes and grown under contained conditions, Council Directive 90/219/EEC⁸ applies. A copy of any written consent or consents of the competent authorities to the use under containment of the genetically modified organisms for research, development and production purposes pursuant to Directive 90/219/EEC must be provided.

Enzymes from a GM source or GM micro-organisms meeting the requirements of Directive 2001/18/EC or 90/219/EEC shall, for the purposes of these guidelines, be treated as any other additive.

2.2.5. Toxins and virulence factors

Strains of micro-organism belonging to a taxonomic group which includes members known to be capable of the production of toxins or other virulence factors, should be subject to appropriate tests to demonstrate at a molecular and cellular level the absence of any cause for concern. In each case, the absence of a functional gene encoding the toxin(s) or other virulence factors should be established. In the specific case of *Bacillus*, use of strains from the *Bacillus cereus* taxonomic group either as the viable micro-organism or as a source of enzymes should be avoided. For other *Bacillus* spp, and in particular those from the *Bacillus subtilis* group, evidence that genes encoding known enterotoxins are absent or, if present, are incomplete and incapable of expressing the active toxin should be provided. In addition,

⁶ OJ EC N° L 106 of 17.04.2001, p. 1.

⁷ OJ EC N° L 322 of 23.11.1991, p. 1.

⁸ OJ EC N° L 117 of 08.05.1990, p. 1.

relevant cytotoxicity tests able to detect the effects of *Bacillus* spp. toxins including the emetic toxin should be included ⁹.

Because of the increased incidence of enterococcal strains as a cause of nosocomial infections, all strains of this genus should be screened for and shown free of genes encoding known virulence determinants.

2.2.6. *Antibiotic production and antibiotic resistance*

Enzyme preparations should be free of antibiotic activities relevant to the use of antibiotics in humans or animals, unless a direct consequence of the catalytic property of the enzyme.

Micro-organisms intended as active agents should not be capable of the production of antimicrobial substances relevant to the use of antibiotics in humans or animals.

Strains of bacteria intended for use as an additive should not contribute further to the reservoir of antibiotic resistance genes already present in the gut flora of animals and the environment. Consequently, all strains of bacteria should be tested for resistance to at least one representative of each of the antibiotic families in use in human and veterinary medicine. Where resistance is detected, the genetic basis of the resistance and the likelihood of transfer of resistance to other gut-inhabiting organisms should be established¹⁰.

2.2.7. *Other relevant properties*

A flow chart describing the production and any purification processes used in the preparation of the active ingredients of the additive should be provided. For products of fermentation, a description of the culture medium, fermentation conditions and downstream processing of the fermentation products should be included. This should also indicate the extent to which spent medium is incorporated into the final product. The information presented here should represent one or more inputs into the overall scheme for the preparation of the additive given under 2.1.6. Details of batch to batch variation and quality control procedures also should be included. Application of the methods required in 2.2.2 to monitor genetic drift should be demonstrated by comparison of production cultures to the strain as originally deposited.

Any historical or existing food or medicinal uses of the active agent(s) should be specified.

⁹ Opinion of the Scientific Committee on Animal Nutrition on the safety of use of *Bacillus* species in animal nutrition expressed on 17 February 2000

¹⁰ Opinion of the Scientific Committee on Animal Nutrition on the criteria for assessing the safety of micro-organisms resistant to antibiotics of human clinical and veterinary importance expressed on 3 July 2001

2.3. Characterisation of the additive: physico-chemical and technological properties

2.3.1. Stability of the additive

The stability, defined in terms of loss of catalytic activity in the case of enzymes and loss of viability in the case of micro-organisms, of each form of the additive on exposure to environmental conditions such as light, temperature, pH, moisture and oxygen should be established. On the basis of this information, the expected shelf life of each form of the additive as marketed should be stated and justified by reference to data from at least three batches of the additive.

Data on the stability of each form of the additive during the preparation and storage of premixtures and feedingstuffs, in particular the stability to anticipated process/storage conditions (heat, moisture, pressure/shear and time) should be provided. This should include possible degradation or decomposition products. The expected shelf life of the treated premix or feedingstuff should be stated and justified by reference to data from at least three batches of treated material.

2.3.2. Other physico-chemical or biological properties

Other appropriate physico-chemical or biological properties such as the ability to obtain homogeneous mixtures in premixtures and feedingstuffs and dust-forming properties should be described.

For enzymes, resistance to degradation or loss of biological activity in the digestive tract of target animals should be assessed by *in vivo* studies or *in vitro* simulations of gut conditions.

2.3.3. Incompatibilities with other feed ingredients

Any incompatibilities with feedingstuffs, other approved additives or with medicinal products should be confirmed or excluded.

2.4. Conditions of use of the additive

Where an additive has significant technological as well as zootechnical effects the claims for each effect has to be separately identified and justified.

2.4.1. Technological additives

The proposed technological use in the manufacture of animal feedingstuffs or its application to the raw materials should be defined.

2.4.2. Zootechnical additives

The proposed mode of use of the additive in animal nutrition (*e.g.* animal species or categories and age group/production stage of animal, type of feedingstuff, and any contra-indications) should be defined.

The proposed method and level of inclusion in premixtures, feedingstuffs or raw materials should be stated. This should include the minimum (and maximum) inclusion levels expressed as c.f.u. (micro-organisms) or units of activity (enzymes) per unit weight of premixture, feedingstuff or feed material.

2.4.3. Safety Data Sheet

The Dossier should include a material safety data sheet as foreseen by Directive 91/155/EEC¹¹ defining and laying down the detailed arrangements for the system of specific information relating to dangerous preparations in implementation of Article 10 of directive 88/379/EEC as amended by Directive 93/112/EEC¹². If necessary, measures for the prevention of occupational risks and means of protection during manufacture, handling, use and disposal should be proposed.

2.5. Control methods

2.5.1. General methods

Description of the methods used for the determination of the criteria listed under items 2.1.3, 2.1.4, 2.1.5, 2.2.5, 2.2.6, 2.3.1, 2.3.2, and 2.3.3 should be provided

2.5.2. Description of the qualitative and quantitative methods for routine control of the active agent in premixtures and feedingstuffs.

For enzymes, this should include methods to recover activities bound to organic matter and should take account of potentially inhibitory compounds in premixtures. For micro-organisms, any use of recovery media before viable counts are made should be described. Each method should specify the sampling method used, its accuracy, precision, limits of detection, limits of quantification and the validation procedure used.

¹¹ OJ EC N° L 76 of 22.03.1991, p. 35.

¹² OJ EC N° L 314 of 16.12.1993, p. 38.

3. SECTION III - STUDIES CONCERNING THE EFFICACY OF THE ADDITIVE

3.1. Technological use

The present major technological use of micro-organisms and enzymes is as agents intended to aid an ensiling process and/or to contribute to silage stability. This application falls outside the scope of Council Directive 70/524/EEC.

For other claims of a technological nature, in line with the requirements for chemical additives, evidence of the efficacy of the additive is required. Each claimed effect and its measurement, preferably using recognised and acceptable methods, under the intended conditions of use and in comparison with appropriate control feedingstuffs should be described. These investigations must be designed and performed so as to permit statistical evaluation.

Full information on the active agents, preparations, premixes and feedingstuffs examined and the treatment and testing conditions employed should be provided. Positive and negative effects, both technological and biological, should be described for each experiment.

3.2. Studies of the effects on animals

Studies on zootechnical additives must be performed in target species/animal categories for which the additive is intended in comparison with negative control groups. Studies must permit the evaluation of the efficacy of the additive according to farming practice in the European Union. Similar protocol designs should where possible be used for all trials so that data can eventually be tested for homogeneity and pooled (if tests so indicate) for added statistical evaluation.

No single design is recommended, flexibility being provided to allow for scientific discretion in the design and conduct of the studies. The experimental design used must be justified according to the claim for the use of the additive and must include consideration of adequate statistical power.

3.2.1. Evidence of efficacy

Claims for microbial and enzyme products can be considered under the following headings:

- improved performance and feed conversion of the target species;
- reduced morbidity or mortality which improves the welfare of the target species. This also may lead to cost-savings for the producer through less veterinary intervention, reduced labour costs or by enabling increased numbers of animals to reach slaughter weight;

- benefits to the consumer through improved product quality (e.g. reduced cholesterol in eggs, reduction in milk fat content, reduced contamination of poultry with human enteropathogens).
- benefits to the wider environment.

Claims for efficacy can fall under one or more headings, but where multiple claims are made each should be separately demonstrated. Whatever the measure of efficacy adopted, a minimum of three studies demonstrating a statistically significant ($P < 0.05$) improvement should be provided. In the case of ruminants, where homogeneity of experimental animals is less easily obtained, a probability of $P < 0.1$ would be acceptable. In addition, data relating to any other trials which failed to reach statistical significance also should be supplied. The three significant studies preferably should be done in at least two different locations.

As a principle, the duration of studies designed to demonstrate the effectiveness of an additive in enhancing some aspect of *animal performance* should be related to the time at which farm output is valued in economic terms and any benefit to the producer demonstrated at this stage. It is insufficient to demonstrate efficacy for only part of a production period, unless the magnitude of the benefit achieved within this period is sufficient to provide the producer with a significant gain when the value of the produce is finally realised^{13 & 14}.

The appropriate duration of studies for common target species/categories is suggested below. If studies are reported which differ in duration from those proposed, then this must be justified. Any revised protocol must adhere to the principle outlined above.

Calves (only fed milk replacer)	Total feeding period until slaughter
Calves (for fattening or rearing)	A minimum of 6 weeks from birth
Cattle for fattening	From 100 kg (or according to local custom until slaughter
Dairy cows (milk production)	A minimum of 100 days. If the first 100 days are used then the remaining lactation period should be included
Dairy cows (if effect on reproduction claimed)	Two cycles
Sheep for fattening	From weaning to slaughter (according to local custom)

¹³ Opinion of the Scientific Committee on Animal Nutrition on the assessment under Council Directive 87/153/EEC of the efficacy of micro-organisms used as feed additives, expressed on 18 February 2000.

¹⁴ Opinion of the Scientific Committee on Animal Nutrition on the assessment of efficacy of enzymes, adopted on 27 April 2000.

Goats for fattening	From weaning to slaughter (according to local custom)
Piglets	Until weaning (creep feed) From weaning to 25 kg (or according to local custom)
Pigs for fattening	Growing/fattening period until slaughter
Sows (if effect on reproduction claimed)	Two cycles
Rabbits for fattening	From weaning until slaughter
Breeding does	At least two cycles
Chickens for fattening	Day 1 to a minimum of 35 days (until slaughter)
Laying hens	A minimum of 24 weeks. If effects are claimed lasting over the whole laying period, then this should be reflected in the duration of the trial
Turkeys for fattening	From day 1 to a minimum of 12 weeks (until slaughter)
Horses for fattening	A minimum of 12 months until slaughter
Fish	One production period to market weight

For other types of claims, the same principle should apply. Thus for improved egg or milk quality, the duration does not have to be the whole laying/milking period since the produce is sold (valued) on a regular basis throughout the period. However, authorisation will not be based on extrapolation of data and will only be for the period for which evidence of an effect is provided. Similarly, claims relating to morbidity or mortality during the perinatal period need only cover this period provided claims are restricted to a reduced veterinary input or the improved welfare of the animal – the benefits being immediate. Claims for improved survivability should be supported by measurement of survival to the time of sale or slaughter.

3.2.2. *Digestion/balance studies*

Digestion/balance studies may be used in support of animal performance studies to provide evidence of mode of action. In some cases, particularly in relation to claims of environmental benefit (*e.g.* effect of phytase on phosphorus bioavailability), efficacy may be better demonstrated by balance studies and may be used in preference to performance trials (see 3.4). Such experiments should use numbers and types of animals appropriate to the claim being made.

3.2.3. *Experimental conditions*

Trials designed to demonstrate the efficacy of the product should be reported individually, giving details of the controls and each experimental treatment.

Trials should ideally be compliant with the criteria established by a recognised, externally-audited, quality assurance scheme. In the absence of such a scheme, evidence should be provided to show that the work was done by qualified personnel using appropriate facilities and equipment and responsible to a named Study Director. Test substances should be well characterised and identified and all study plans, raw data and final reports should be archived.

The trial protocol should be carefully drawn up by the Study Director with regard to general descriptive data as follows and any subsequent amendments documented: Herd: location and size; feeding and rearing conditions. For aquatic species, size and number of tanks or pens at the farm and water quality.

3.2.3.1. Animals: species (aquatic species intended for human consumption should be identified by their colloquial name followed in parenthesis by their Latin binomial), breed, age, sex, initial weight, identification procedure, physiological stage and general health.

3.2.3.2. Diets: description of manufacture and quantitative composition in terms of ingredients used, relevant nutrients (analysed values) and energy. Feed intake records.

3.2.3.3. Concentration of the active substance in the feedingstuffs - established by a control analysis using the appropriate recognised method.

3.2.3.4. Date and exact duration of testing. Date and nature of the examinations performed.

3.2.3.5. The timing and prevalence of any undesirable consequences of treatment in individuals or groups must be reported (give details of the observation programme used in the study).

3.2.3.6. All additives studied under farm conditions must have good scientific evidence of safety for the user, consumer, animal and the environment. Where an additive does not meet the requirements for consumer safety any study undertaken should be designed to prevent animal products derived from the test animals from entering the human food chain.

3.2.4. *Efficacy of multicomponent additives*

For microbial additives comprised of two or more strains of micro-organisms, evidence of efficacy should be produced for the complete additive at the lowest dose level claimed.

Enzyme additives may contain multiple activities arising from a single fermentation and a single producer strain, or from a blend of fermentation products involving one or more producer strains. For additives containing the product of a single fermentation, evidence of efficacy should be produced for the complete additive at the lowest dose level claimed. For products

consisting of a blend of the products of two or more fermentations, provided the efficacy of each fermentation product has been separately assessed and demonstrated and provided that the nature of the claim is identical, then the efficacy of the blend can be assumed. If the efficacy of one or more fermentation products in a blend is unknown or cannot be separately assessed then evidence of efficacy should be produced for the additive at the lowest dose level claimed.

3.3. Studies on the quality of animal produce

Where an effect on the quality of animal products is claimed for the additive, efficacy should be demonstrated to the same level of stringency as for effects on performance characteristics. This would similarly require the use of animal studies, which should be completed to the standards outlined in 3.2. In cases when the claimed properties of the additive do not include effects on product quality, examination of animal products for organoleptic, nutritional, hygienic and technological qualities may be required to confirm the absence of any deleterious effects.

3.4. Studies on the effects on the characteristics of animal wastes

If the additive is intended to modify some characteristics of animal waste (e.g. reduced nitrogen or phosphorus concentration, odour, or volume), then studies demonstrating these properties are required. Such studies should satisfy the criteria outlined in 3.2 with respect to the number and nature of such studies, their appropriateness to European agriculture/aquaculture and the statistical treatment of data. Evidence from digestion/balance studies (section 3.2.2) with the target species may give support to animal production studies and, in some cases, may substitute.

4. SECTION IV - STUDIES CONCERNING THE SAFETY OF USE OF THE ADDITIVE

The studies outlined in this section are intended to permit assessment of:

- the safety of use of the additive in the target species;
- any risk associated with the increased persistence and shedding of enteropathogens;
- any risks to the consumer of foods derived from animals given feedingsuffs containing or treated with the additive;
- the risks from respiratory, other mucosal, eye or cutaneous contact for persons likely to handle the additive or premixtures or complete feedingsuffs containing the additive;
- the risks of adverse effects on the environment, from the additive itself or by products derived from the additive and excreted by animals;

A more limited submission will normally be accepted for a proposed extension of the authorised use to a minor species that is physiologically and metabolically proximate to one in which the use of the additive has already been approved.

Knowledge of the composition and of the physico-chemical and biological properties of the major excreted materials deriving from the additive are required to define the extent of the studies necessary for assessment of the risk of adverse effects on the environment or persistence in the environment.

4.1. Studies on target species

4.1.1. Tolerance tests on target species/animal categories

The aim of the tolerance test is to establish that no unfavourable effects occur when the final product (not just the active agent(s)) is consumed at a minimum of ten times the maximum recommended dose. Such a tolerance test must be conducted with each of the target species/animal categories for which a claim is made. A test period of one month is acceptable for young, fast-growing animals but for adults, such as dairy cattle in lactation, three months is the minimum period considered adequate. Application rates greater than ten-fold may be used but not to reduce the recommended duration of the test period.

Test animals, after a short period of adaptation, should be routinely monitored for visual evidence of adverse clinical effects, for performance characteristics, for product quality where relevant, for routine blood chemistry and for any other parameters necessary to ascertain effects on target animal health. Any adverse effects detected during efficacy trials should also be reported in this section.

4.1.2. *Effects on the microflora of the digestive tract.*

Studies on the effects of the additive on the microflora of digestive tract are required if the additive consists of micro-organisms or, in the case of enzymes, when a claim is made concerning or implying an effect on intestinal microflora or when the enzymatic activity makes such an effect likely. In most cases, it is sufficient to limit such studies to the enumeration of microbial groups that can be routinely cultivated from the faecal flora and have safety implications (*i.e.* opportunistic pathogens including coliforms, enterococci and clostridia). A more specific or comprehensive analysis of the induced changes in the microbial composition of the gut may be required when effects are restricted to particular sites (*e.g.* the rumen) or when the health or wellbeing of the target species is in question.

Intestinal survival and rate of disappearance from faeces after withdrawal of the particular strain(s) used in microbial additives should be established. For gut-derived strains this will require the development of reliable means of distinguishing the added strain(s) from endogenous intestinal species.

Additional studies may be required, depending on the nature of the additive, the associated claims and the target species. Tests on the excretion of human enteropathogens (*e.g.* *E.coli*, *Salmonella* spp., *Campylobacter* spp.) would be required where a specific claim is made for pathogen control. Such studies also are required if the target species is a companion animal because of the risk to owners.

4.1.3. *Metabolism and residue studies*

Metabolism and residue studies will not normally be required for enzyme and microbial additives.

4.2. **Consumer safety assessment**

The enzymes and micro-organism used in feed additives are not expected to be harmful *per se*. However, enzymes or micro-organisms form only a small part of the complete additive, which, in most cases, will include ill-defined components originating from the fermentation or possibly from the carrier. Consequently, it is necessary to test the additive if used in food-producing animals to ensure that it does not contain mutagenic or otherwise toxic materials that may be passed on to human consumers of foods derived from animals given feedingstuffs treated with these products.

The safety of human consumers of foods derived from animals given feed treated with enzyme or microbial products should be assured by testing the fermentation broth or fraction of the fermentation broth concentrate of enzymes that is used in the commercial product. A battery of genotoxicity tests and sub-chronic oral toxicity tests should be used. For microbial products, living micro-organisms may interfere and, where necessary, these should be removed from the test material prior to toxicological testing.

The non-active ingredients in the product (excipients, bulking material, etc.) should be shown to be safe. Usually they will be accepted feed ingredients

or other materials that are recognised as being of no or low toxicity. It will be necessary to provide evidence of the safety of any novel ingredients that may be used.

4.2.1. *Genotoxicity studies including mutagenicity*

In order to identify the presence of genotoxic contaminants or metabolites in products based on micro-organisms or enzymes, a selected combination of at least two different genotoxicity tests must be used. The test methods must be internationally validated and should be performed in accordance with EU or OECD methodological Guidelines. The initial test battery should normally include a bacterial reverse mutation assay and an *in vitro* assay for clastogenicity in mammalian cells (e.g. a metaphase cytogenetics assay). The tests should be performed in the presence and absence of metabolic activation.

If the results of *in vitro* genotoxicity tests indicate a genotoxic potential, it may be possible to perform further studies to demonstrate that there is no genotoxic hazard with *in vivo* exposure. It will normally be necessary to demonstrate an absence of mutagenicity at two different somatic tissue sites in mutagenicity studies performed *in vivo* in mammals. A product that is genotoxic *in vivo* would be regarded as unacceptable

4.2.2. *Oral toxicity studies*

The duration of the tests must be at least 90 days. The preferred mode of administration is by incorporation into the feed, but if this is impractical, administration in drinking water or by oral gavage may be used. For additives intended for use in food-producing animal species, the studies should be carried out in at least one laboratory species (usually the rat). Studies should be performed according to EU or OECD Guidelines for rodents or for non-rodents.

Oral toxicity studies are not required for additives intended for use only in animals not producing food for human consumption.

4.3. Worker safety assessment

The worker safety of the formulated product should be addressed. Contamination of skin and/or inhalation are the most likely routes of exposure. It may be necessary to separately consider exposure during different activities, such as production of the additive, incorporating the additive into feed and handling the mixed feed.

4.3.1. *Irritancy*

The formulated commercial product should be examined for irritancy using validated laboratory animal tests for skin irritation and for eye irritation (liquid products).

4.3.2. Skin sensitisation

Pure enzymes and micro-organisms are unlikely to penetrate intact healthy skin and as such are unlikely to cause skin sensitisation. However, products based on enzymes and micro-organisms are often chemically ill-defined and contain substances other than the enzymes and micro-organisms as excipients, metabolites, contaminants, etc. Consequently, it is necessary to test for skin sensitisation potential in case any of the components can penetrate the skin and cause sensitisation. However it is recognised that there are difficulties for testing enzymes and micro-organisms, therefore the filtered aqueous extract of solid products could be used.

4.3.3. Toxic effects on the respiratory system

Enzyme and microbial additives will be regarded as respiratory sensitisers (R42) unless convincing evidence to the contrary is provided. As such, measures should be taken to minimise the inhalation exposure of workers and inhalation toxicity studies will thus not be required. However, data on the dusting potential and particle size distribution of the product should be provided to give an indication of whether a free-flowing solid product is likely to form a dust that can be inhaled into the lungs. This should be supported by measurement of concentrations in air at the workplace. Inhalation toxicity studies will not be required unless components of the final product other than the active agents give cause for concern.

4.3.4. Systemic toxicity

The toxicity data generated to meet consumer safety and other requirements may be relevant to the evaluation of worker safety and should be taken into consideration.

4.3.5. Control measures

Product reformulation or modification of the recommended conditions for use and disposal of the product are preferred solutions to the management of any risks associated with enzyme and microbial products. Use of personal protective equipment alone should be regarded as a measure of last resort.

4.4. Environmental risk assessment

The impact on the environment of enzyme and microbial additives can be considered negligible and to require assessment only in exceptional cases.

4.4.1. Enzyme additives

Enzymes are proteins and are largely degraded in the upper digestive tract of animals like any other dietary source of protein. A limited amount of the added activity may be protected by the organic fraction of the diet and so can reach the lower digestive tract to be excreted. However, enzymes with the same catalytic function as many added activities are also produced by bacteria naturally present in the lower digestive tract. Consequently, any added activity escaping digestion represents only a small fraction of that naturally and continuously produced in healthy animals. Enzymes active

against feed ingredients and added to rations are not expected to have detectable effect on organic matter in the soil or in the watercourse.

4.4.2. Microbial additives

The majority of micro-organisms selected for use in microbial additives are of gut origin, often isolated from the digestive tract of the principal target species, or derive from soil. The numbers of such organisms added to the diet of animals in the form of an additive have no significant effect on the total numbers of micro-organisms excreted, although they may increase the proportion of one or more of the species deposited. In such cases a neutral effect on the environment can be assumed. An impact assessment would be required only if the organism(s) were not of gut origin and were not already ubiquitous in the environment.

4.4.3. Genetically modified micro-organisms

A GM micro-organism within the meaning of article 2(1) and 2(2) of Council Directive 2001/18/EC must first satisfy the requirements of the release Directive, which includes an assessment of any risks for the environment related to the GMO(s) contained in the product. Micro-organisms satisfying the requirements of 2001/18/EC are treated as any other micro-organism (see 4.4.2).

5. SECTION V - FORM OF MONOGRAPH

The monograph is intended to provide a brief description of the product and the information necessary to establish its safety. Details of results and experimental methods described in the dossier are not required.

5.1. Identity of the additive

- (1) Proposed proprietary name(s).
- (2) Type of additive according to its main function.
- (3) Qualitative and quantitative composition (active agents and other components). Each strain of micro-organism must be described separately and the numbers of each strain in the final product given as colony forming units (c.f.u.) per unit weight. For enzymes, each declared activity should be described and the number of units of each activity in the final product provided.
- (4) Qualitative and quantitative composition of any impurities (contaminating micro-organisms, heavy metals, toxins).
- (5) Physical state of each form of the product. For solid preparations, particle size distribution, dusting potential and the use of processes such as encapsulation which affect the physical properties. For liquid preparations, viscosity and bulk density values.
- (6) Manufacturing process including any specific processing procedures.

5.2. Specifications concerning the active agent(s)

- (1) The name and taxonomic status of each micro-organism according to the latest published information in the international Codes of Nomenclature. Commonly used and accepted synonyms. The number and systematic name proposed by the International Union of Biochemistry (IUB) for enzymes or a systematic name consistent with the IUB rules of nomenclature. Any trivial names used should be related to the systematic name at first mention.
- (2) Biological origin of all active agents. Evidence of deposition of micro-organisms (whether product or producer strain) in the form of a certificate of deposition from a recognised culture collection. All relevant morphological, physiological, and molecular characteristics necessary to identify the strain and confirm genetic stability.
- (3) For enzymes produced from a GM source and for GM micro-organisms, the identity and description of recipient and donor organism with particular reference to any characteristics likely to cause concern. For GM micro-organisms, the nature and purpose of the genetic modification.

- (4) Evidence of compliance with the release Directive for GMOs (GM micro-organisms) or use under containment (enzyme from a GM source).
- (5) Evidence of the absence of functioning genes encoding toxin(s) or other virulence factors for all strains of micro-organisms belonging to a taxonomic group which includes members known to be capable of their production.
- (6) Evidence of the absence of antibiotic activities relevant to use of antibiotics in humans or animals in enzyme preparations unless a direct consequence of the catalytic property of the enzyme. Micro-organisms should not be capable of the production of antibiotics relevant to the use of antibiotics in humans or animals. Screen of each microbial strain contributing to the additive for resistance to at least one representative of each of the antibiotic families in use in human and veterinary medicine. Where resistance is detected, the genetic basis of the resistance and the likelihood of transfer of resistance to other gut-inhabiting organisms.
- (7) Other relevant properties. A flow chart describing the production and any purification processes used in the preparation of the active ingredients of the additive. For products of fermentation, a description of the culture medium, fermentation conditions and downstream processing of the fermentation products and extent to which spent medium is incorporated into the final product. Details of batch to batch variation and quality control procedures. Any historical or existing food or medicinal uses of the active agent(s).

5.3. Physico-chemical, technological and biological properties of the additive

- (1) Stability of the additive, defined in terms of loss of catalytic activity of an enzyme or loss of viability of a micro-organism, on exposure to environmental conditions such as light, temperature, pH, moisture and oxygen. Proposal of a shelf life.
- (2) Stability during the preparation of premixtures and feedingstuffs, in particular stability to anticipated process conditions (heat, moisture, pressure/shear and time).
- (3) Stability during the storage of premixtures and processed feedingstuffs under defined conditions. Proposal of a shelf life.
- (4) Other appropriate physico-chemical, technological or biological properties such as dispersability under favourable conditions in order to obtain and keep homogeneous mixtures in premixtures and feedingstuffs, antidusting properties, dispersability in liquids. Evidence of survival in an active state in the gastrointestinal tract.
- (5) Incompatibilities with feedingstuffs, other approved additives or with medicinal products. Effect of metal ions on enzyme activity.

5.4. Control methods

- (1) Description of the methods used for the determination of the criteria listed under items 2.1.3, 2.1.4, 2.1.5, 2.2.5, 2.2.6, 2.3.1, 2.3.2, and 2.3.3. If the said methods have been published, literature references may suffice and the corresponding reprints provided. Each method should specify the sampling method used, its accuracy, precision, limits of detection, limits of quantification and the validation procedure used.
- (2) Description of the qualitative and quantitative methods for routine control of the active agent in premixtures and feedingstuffs. Methods to recover enzyme activities bound to organic matter. Use of recovery media for microbial counts.

5.5. Biological properties of the additive

- (1) For zootechnical additives particulars of the effects on: 1) performance and feed conversion of the target species; 2) morbidity or mortality, cost-savings for the producer through less veterinary intervention, reduced labour costs and numbers of animals reaching sale or slaughter weight; 3) product quality and 4) the wider environment.
- (2) For technological additives, relevant technological effects.
- (3) Any adverse effects, contra-indications or warnings, including biological interactions, with particulars of their justification.

5.6. Other characteristics suitable for identification of the additive

6. SECTION VI - FORM OF IDENTIFICATION NOTE

1. Identity of the additive
 - 1.1 Type of additive
 - 1.2 Physical state
 - 1.3 Qualitative and quantitative composition
 - 1.4 Community registration number (EC number)
 - 1.5 Packaging
2. Specifications concerning the active agent
 - 2.1 Nomenclature
 - Systematic and trivial name
 - IUB number (enzyme)
 - Culture collection and deposition number (micro-organism)
 - 2.2 Biological origin of each enzyme activity
3. Physico-chemical, technological and biological properties of the additive
 - 3.1 Stability of additive
 - 3.2 Stability during the preparation of premixtures and feedingstuffs
 - 3.3 Stability during storage of premixtures and feedingstuffs
 - 3.4 Other properties
4. Conditions of use
 - 4.1 Species or category of animals, maximum age if specified
 - 4.2. Minimum and maximum content in feedingstuffs
 - 4.3. Contra-indications
 - 4.4. Warnings, including the risk phrase “**R42** May cause sensitisation by inhalation”
5. Person responsible for putting into circulation
 - 5.1 Name
 - 5.2. Address
 - 5.3. Registration number
6. Manufacturer
 - 6.1. Name
 - 6.2. Address
 - 6.3. Approval number or registration number assigned to the establishment or the intermediary.