European Food Safety Authority

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SCIENTIFIC OPINION

Scientific Opinion on DL-methionine, DL-methionine sodium salt, the hydroxy analogue of methionine and the calcium salt of methionine hydroxy analogue in all animal species; on the isopropyl ester of methionine hydroxy analogue and DL-methionine technically pure protected with copolymer vinylpyridine/styrene in dairy cows; and on DL-methionine technically pure protected with ethylcellulose in ruminants[1]

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP)[2,3]

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

Methionine is an essential amino acid in all animal species. Supplementation of appropriate amounts of methionine and analogues to meet requirements is safe for the target species. The use of methionine-based additives will not result in an increased content in tissues and products. Residues present in animal tissues and products resulting from the extremely small quantities of free styrene and 2-vinylpyridine found in DL-methionine copolymer (DL-Met-cop) are very unlikely to raise concerns about consumer safety. None of the products are considered to present a significant inhalation risk. DL-Methionine (DL-Met), DL-Met-cop and DL-methionine protected with ethylcellulose (DL-Met-ec) are non-irritant to skin and eyes, and the lack of sensitisation potential demonstrated for DL-Met is considered relevant to DL-Met-cop and DL-Met-ec. DL-Methionine sodium salt (DL-Met-Na) is considered to be corrosive to skin and eyes. The hydroxy analogue of methionine (HMTBa) is an irritant to the skin and corrosive to the eyes. The calcium salt of HMTBa, HMTBa-Ca, is irritant to the eyes. The absence of dermal sensitation potential demonstrated for HMTBa is considered relevant for HMTBa-Ca. The isopropyl ester of HMTBa, HMTB-i, is non-irritant to skin/eyes, and is not a dermal sensitiser. The use of these products as feed additives does not represent a risk to the environment. DL-Met, DL-Met-Na, HMTBa and HMTBa-Ca are effective dietary sources of methionine for protein synthesis in monogastric animals (including fish). The hydroxy analogues show a somewhat lower bioefficacy than the DL-methionine forms. They can all be used in ruminants, particularly when protected against ruminal degradation by specific formulations. DL-Met-cop, HMTB-i and DL-Met-ec escape ruminal degradation to a certain extent, the remainder being available for gastrointestinal absorption. The FEEDAP Panel does not support the use of the additives under application in water for drinking because of concerns about target animal safety and the maintenance of optimum performance.

[1] On request from the European Commission, Question No EFSA-Q-2010-00995, adopted on 6 March 2012.

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KEY WORDS

DL-Methionine, DL-methionine sodium salt, hydroxy analogue of methionine, calcium salt of methionine hydroxy analogue, isopropyl ester of methionine hydroxy analogue, DL-methionine protected with copolymer vinylpyridine/styrene, DL-methionine protected with ethylcellulose, safety, efficacy

SUMMARY

Following a request from the European Commission, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on DL-methionine (DL-Met), DL-methionine sodium salt (DL-Met-Na), the hydroxy analogue of methionine (HMTBa) and the calcium salt of the methionine hydroxy analogue (HMTBa-Ca) in all animal species; on DL-methionine technically pure protected with copolymer vinylpyridine/styrene (DL-Met-cop) and the isopropyl ester of methionine hydroxy analogue (HMTB-i) in dairy cows; and on DL-Met technically pure protected with ethylcellulose (DL-Met-ec) in ruminants.

Methionine is an essential amino acid in all animal species. Methionine is clearly recognised as the first limiting amino acid in poultry, and probably also in high-yielding cows, and as the second or third limiting amino acid in pigs fed conventional diets. Supplementation of appropriate amounts of methionine and methionine analogues to meet requirements is safe for the target species. The earliest signs of a critical overdose are reduced feed intake and weight gain. The level of supplementary methionine and its analogues that is toxic varies from species to species and depends on the basal diet and its content of sulphur-containing amino acids.

Methionine from DL-Met and its hydroxy analogue and their salts is incorporated in the protein of tissues and products, showing a constant amino acid pattern. Consequently, the use of these additives will not result in an increased methionine content in tissues and products. Doses exceeding the requirement will be excreted.

Residues present in the animal tissues and products resulting from the extremely small quantities of free styrene and 2-vinylpyridine found in DL-Met-cop are very unlikely to raise concerns about consumer safety.

None of the products are considered to present a significant inhalation risk. DL-Met, DL-Met-cop and DL-Met-ec are considered non-irritant to skin and eyes, and the lack of sensitisation potential demonstrated for DL-Met is considered to apply also to DL-Met-cop and DL-Met-ec. DL-Met-Na is considered corrosive to skin and eyes and harmful if swallowed. HMTBa is an irritant to the skin and corrosive to the eyes but is not a dermal sensitiser. HMTBa-Ca is irritant to the eyes but not to the skin. The absence of a dermal sensitisation potential demonstrated for HMTBa is considered to apply also to HMTBa-Ca. HMTB-i is non irritant to the skin and eyes, and it is not a dermal sensitiser.

The use of these products as feed additives does not represent a risk to the environment.

DL-Met and its sodium salt as well as HMTBa and its calcium salt are effective dietary sources of methionine for protein synthesis in monogastric animals (including fish). The hydroxy analogues show a somewhat lower bioefficacy than the DL-methionine forms. All of them can also be used in ruminants, particularly when they are administered in specially formulated additives or complementary feedingstuffs to protect them from degradation by the ruminal microbiota. The coated and otherwise protected methionine forms, DL-Met-cop, HMTB-i and DL-Met-ec, escape ruminal degradation to a certain extent, the remainder being available for gastrointestinal absorption. However, product identity of the DL-Met-cop used in studies with the product under application has not been demonstrated.

Because of concerns about target animal safety and maintenance of optimum performance, the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) strongly recommends that the use of all methionine forms under application in water for drinking is not authorised.

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BACKGROUND

Regulation (EC) No 1831/2003[4] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7 and Article 10(2) of that Regulation also specify that for existing products within the meaning of Article 10(1), an application shall be submitted in accordance with Article 7, at the latest one year before the expiry date of the authorisation given pursuant to Directive 70/524/EEC for additives with a limited authorisation period, and within a maximum of seven years after the entry into force of this Regulation for additives authorised without time limit or pursuant to Directive 82/471/EEC.

The European Commission received a request from the company AMAC/EEIG – Amino Acids Authorisation Consortium[5] for authorisation and re-evaluation of the products DL-Methionine, DL-Methionine sodium salt, Hydroxy Analogue of Methionine, Calcium salt of Methionine Hydroxy Analogue, Isopropyl ester of Methionine Hydroxy Analogue when used as a feed additive for all animal species and DL-Methionine technically pure protected with copolymer vinylpyridine/styrene when used as a feed additive for dairy cows, DL-Methionine technically pure protected with ethylcellulose when used as a feed additive for ruminants (category: nutritional additives; functional group: amino acids, their salts and analogues) under the conditions mentioned in Table 1.

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 4(1) (authorisation of a feed additive or new use of a feed additive) and under Article 10(2)/(7) (re-evaluation of an authorised feed additive). EFSA received directly from the applicant the technical dossier in support of this application.[6] According to Article 8 of that Regulation, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. The particulars and documents in support of the application were considered valid by EFSA as of 07 October 2011

The additive DL-Methionine technically pure protected with ethylcellulose when used as a feed additive for ruminants is currently not authorised in the European Union.

The additives DL-Methionine, DL-Methionine sodium salt, Hydroxy Analogue of Methionine, calcium salt of Methionine hydroxy analogue, when used as a feed additive for all animal species; Isopropyl ester of Methionine hydroxy analogue and DL-Methionine technically pure protected with copolymer vinylpyridine/styrene when used as a feed additive for dairy cows are permanently authorised for use for all animal species and dairy cows, respectively.

According to Commission Directive 2006/141/EC on infant formulae and follow-on formulae, amino acids as L-methionine may be used in the manufacture of infant formulae and follow-on formulae in order to satisfy the requirements on amino acids and other nitrogen compounds.[7]

L-methionine is described in the European Pharmacopeia, monograph 01/2008:0624. Methionine is "generally recognised as safe" pharmacological active substance for all food producing species in annex II of Commission Regulation (EC) No 1931/1999[8], amending Annexes I, II and III of Council Regulation (EEC) No 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin.

[4] Official Journal L 268, 18.10.2003, p. 29.
[5] AMAC/ EEIG Amino Acids Authorisation Consortium – Avenue Louise 120, Box 13 – 1010 – Brussels, Belgium.
[6] EFSA Dossier reference: FAD-2010-0023.
[7] Official Journal L 401, 30.12.2006, p 1-33.
[8] Official Journal L 240, 10.09.1999, p 3-10.

DL-methionine is included in the Community list of flavouring substances as FL. No. 17.014.

Methionine is registered as an ingredient for use in cosmetics as antistatic and for skin conditioning (Commission Decision 2006/257/EC).[9]

TERMS OF REFERENCE

According to Article 8 of Regulation (EC) No 1831/2003, EFSA shall determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the safety for the target animal(s), consumer, user and the environment and the efficacy of the product DL-Methionine, DL-Methionine sodium salt, Hydroxy Analogue of Methionine, Calcium salt of Methionine Hydroxy Analogue for all animal species, Isopropyl ester of Methionine Hydroxy Analogue and DL-Methionine technically pure protected with copolymer vinylpyridine/styrene when used as a feed additive for dairy cows, DL-Methionine technically pure protected with ethylcellulose when used as a feed additive for ruminants, when used under the conditions described in Table 1.

[9] Official Journal L 97, 5.4.2006, p. 1–528

Table 1. Description and conditions of use of the additive as proposed by the applicant

Additive	Methionine / DL-methionine, technically pure			
Registration number/EC No/No (if appropriate)	3.1.1			
Category(ies) of additive	1. Nutritional additives			
Functional group(s) of additive	(c) amino acids, their salts and analogues			
Description				
Composition, description	Chemical formula	Purity criteria (if appropriate)	Method of analysis (if appropriate)	
DL-Methionine	$C_5H_{11}NO_2S$	min. 99 %	AOAC 999:13 and Reg. (EC) N° 152/2009	
Trade name (if appropriate)	-			
Name of the holder of authorisation (if appropriate)	-			
Conditions of use				
Species or category of animal	Maximum Age	Minimum content	Maximum content	Withdrawal period (if appropriate)
		mg or Units of activity or CFU/kg of complete feedingstuffs (select what applicable)		
All animal species or categories	-	-	-	-
Other provisions and additional requirements for the labelling				
Specific conditions or restrictions for use (if appropriate)	To be used also via water for drinking			
Specific conditions or restrictions for handling (if appropriate)	-			
Post-market monitoring (if appropriate)	-			
Specific conditions for use in complementary feedingstuffs (if appropriate)	-			
Maximum Residue Limit (MRL) (if appropriate)				
Marker residue	Species or category of animal	Target tissue(s) or food products	Maximum content in tissues	
-	-	-	-	

Additive	Methionine / Concentrated liquid sodium DL-methionine technically pure			
Registration number/EC No/No (if appropriate)	3.1.4			
Category(ies) of additive	1. Nutritional additives			
Functional group(s) of additive	(c) amino acids, their salts and analogues			
Description				
Composition, description	Chemical formula	Purity criteria (if appropriate)	Method of analysis (if appropriate)	
DL-Methionine sodium salt	$(C_5H_{10}NO_2S)Na$	min. 40 % of DL-Methionine	AOAC 999:13 and Reg. (EC) N° 152/2009	
Trade name (if appropriate)	-			
Name of the holder of authorisation (if appropriate)	-			
Conditions of use				
Species or category of animal	Maximum Age	Minimum content	Maximum content	Withdrawal period (if appropriate)
		mg or Units of activity or CFU/kg of complete feedingstuffs (select what applicable)		
All animal species or categories	-	-	-	-
Other provisions and additional requirements for the labelling				
Specific conditions or restrictions for use (if appropriate)	To be used also via water for drinking. Not used via premix.			
Specific conditions or restrictions for handling (if appropriate)	-			
Post-market monitoring (if appropriate)	-			
Specific conditions for use in complementary feedingstuffs (if appropriate)	-			
Maximum Residue Limit (MRL) (if appropriate)				
Marker residue	Species or category of animal	Target tissue(s) or food products	Maximum content in tissues	
-	-	-	-	

Additive	Methionine / DL-methionine, technically pure protected with copolymer vinylpyridine/styrene			
Registration number/EC No/No (if appropriate)	3.1.5			
Category(ies) of additive	1. Nutritional additives			
Functional group(s) of additive	(c) amino acids, their salts and analogues			
Description				
Composition, description	Chemical formula	Purity criteria (if appropriate)	Method of analysis (if appropriate)	
DL-Methionine technically pure protected with copolymer vinylpyridine/styrene	$C_5H_{11}NO_2S$	min. 74 % of DL-Methionine	AOAC 999:13 and Reg. (EC) N° 152/2009	
Trade name (if appropriate)	-			
Name of the holder of authorisation (if appropriate)	-			
Conditions of use				
Species or category of animal	Maximum Age	Minimum content	Maximum content	Withdrawal period (if appropriate)
		mg or Units of activity or CFU/kg of complete feedingstuffs (select what applicable)		
Dairy cows	-	-	-	-
Other provisions and additional requirements for the labelling				
Specific conditions or restrictions for use (if appropriate)	-			
Specific conditions or restrictions for handling (if appropriate)	-			
Post-market monitoring (if appropriate)	-			
Specific conditions for use in complementary feedingstuffs (if appropriate)	-			
Maximum Residue Limit (MRL) (if appropriate)				
Marker residue	Species or category of animal	Target tissue(s) or food products	Maximum content in tissues	
-	-	-	-	

Additive	Methionine / DL-Methionine technically pure protected with ethylcellulose			
Registration number/EC No/No (if appropriate)	-			
Category(ies) of additive	1. Nutritional additives			
Functional group(s) of additive	(c) amino acids, their salts and analogues			
Description				
Composition, description	Chemical formula	Purity criteria (if appropriate)	Method of analysis (if appropriate)	
DL-Methionine technically pure protected with ethylcellulose	$C_5H_{11}NO_2S$	min. 85 % of DL-Methionine	AOAC 999:13 and Reg. (EC) N° 152/2009	
Trade name (if appropriate)	-			
Name of the holder of authorisation (if appropriate)	-			
Conditions of use				
Species or category of animal	Maximum Age	Minimum content	Maximum content	Withdrawal period (if appropriate)
		mg or Units of activity or CFU/kg of complete feedingstuffs (select what applicable)		
Ruminants	-	-	-	-
Other provisions and additional requirements for the labelling				
Specific conditions or restrictions for use (if appropriate)	-			
Specific conditions or restrictions for handling (if appropriate)	-			
Post-market monitoring (if appropriate)	-			
Specific conditions for use in complementary feedingstuffs (if appropriate)	-			
Maximum Residue Limit (MRL) (if appropriate)				
Marker residue	Species or category of animal	Target tissue(s) or food products	Maximum content in tissues	
-	-	-	-	

Additive	Methionine / Hydroxy analogue of methionine (HMTBa)			
Registration number/EC No/No (if appropriate)	3.1.6			
Category(ies) of additive	1. Nutritional additives			
Functional group(s) of additive	(c) amino acids, their salts and analogues			
Description				
Composition, description	Chemical formula	Purity criteria (if appropriate)	Method of analysis (if appropriate)	
Hydroxy Analogue of Methionine	$C_5H_{10}O_3S$	min. 88 %	Titration and HPLC	
Trade name (if appropriate)	-			
Name of the holder of authorisation (if appropriate)	-			
Conditions of use				
Species or category of animal	Maximum Age	Minimum content	Maximum content	Withdrawal period (if appropriate)
		mg or Units of activity or CFU/kg of complete feedingstuffs (select what applicable)		
All animal species or categories	-	-	-	-
Other provisions and additional requirements for the labelling				
Specific conditions or restrictions for use (if appropriate)	To be used also via water for drinking. Not used via premix.			
Specific conditions or restrictions for handling (if appropriate)	-			
Post-market monitoring (if appropriate)	-			
Specific conditions for use in complementary feedingstuffs (if appropriate)	-			
Maximum Residue Limit (MRL) (if appropriate)				
Marker residue	Species or category of animal	Target tissue(s) or food products	Maximum content in tissues	
-	-	-	-	

Additive	Methionine / Calcium salt of hydroxyl analogue of methionine (HMTBa-Ca)			
Registration number/EC No/No (if appropriate)	3.1.7			
Category(ies) of additive	1. Nutritional additives			
Functional group(s) of additive	(c) amino acids, their salts and analogues			
Description				
Composition, description	Chemical formula	Purity criteria (if appropriate)	Method of analysis (if appropriate)	
Calcium salt of Methionine Hydroxy Analogue	$C_{10}H_{18}O_6S_2Ca$	min. 84 % of methionine hydroxy analogue	Titration and HPLC	
Trade name (if appropriate)	-			
Name of the holder of authorisation (if appropriate)	-			
Conditions of use				
Species or category of animal	Maximum Age	Minimum content	Maximum content	Withdrawal period (if appropriate)
		mg or Units of activity or CFU/kg of complete feedingstuffs (select what applicable)		
All animal species or categories	-	-	-	-
Other provisions and additional requirements for the labelling				
Specific conditions or restrictions for use (if appropriate)	-			
Specific conditions or restrictions for handling (if appropriate)	-			
Post-market monitoring (if appropriate)	-			
Specific conditions for use in complementary feedingstuffs (if appropriate)	-			
Maximum Residue Limit (MRL) (if appropriate)				
Marker residue	Species or category of animal	Target tissue(s) or food products	Maximum content in tissues	
-	-	-	-	

Additive	Methionine / Isopropyl ester of the hydroxylated analogue of methionine (HMBi)			
Registration number/EC No/No (if appropriate)	3.1.8			
Category(ies) of additive	1. Nutritional additives			
Functional group(s) of additive	(c) amino acids, their salts and analogues			
Description				
Composition, description	Chemical formula	Purity criteria (if appropriate)	Method of analysis (if appropriate)	
Isopropyl ester of Methionine Hydroxy Analogue	$C_8H_{16}O_3S$	min. 95 %	HPLC	
Trade name (if appropriate)	-			
Name of the holder of authorisation (if appropriate)	-			
Conditions of use				
Species or category of animal	Maximum Age	Minimum content	Maximum content	Withdrawal period (if appropriate)
		mg or Units of activity or CFU/kg of complete feedingstuffs (select what applicable)		
Dairy cows	-	-	-	-
Other provisions and additional requirements for the labelling				
Specific conditions or restrictions for use (if appropriate)	Not used via premix			
Specific conditions or restrictions for handling (if appropriate)	-			
Post-market monitoring (if appropriate)	-			
Specific conditions for use in complementary feedingstuffs (if appropriate)	-			
Maximum Residue Limit (MRL) (if appropriate)				
Marker residue	Species or category of animal	Target tissue(s) or food products	Maximum content in tissues	
-	-	-	-	

ASSESSMENT

This opinion is based in part on data provided by a consortium of companies involved in the production/distribution of different forms of methionine and its hydroxy analogues. It should be recognised that these data cover only a fraction of existing additives containing methionine and/or its hydroxy analogues. The application is for the active substance, and the composition of the additive formulations is not the subject of the application. The Panel has sought to use the data provided together with data from other sources to deliver an opinion.

This application contains data from four companies involved in the production of a total of four additives containing DL-methionine as the active substance and of three additives containing the hydroxy analogue of methionine as the active substance.

1. Introduction

Methionine is an essential amino acid in all animal species. Methionine is clearly recognised as the first limiting amino acid in poultry, and probably also in high-yielding cows, and as the second or third limiting amino acid in pigs fed conventional diets. Therefore, additives containing DL-methionine or the hydroxy analogue of methionine as the active substance are frequently used in the feed industry to adjust dietary methionine to meet the requirements of target animals (and fish) in order to maximise production performance and reduce nitrogen emission.

DL-Methionine technically pure (DL-Met), DL-methionine sodium salt (DL-Met-Na), the hydroxy analogue of methionine (HMTBa), the calcium salt of methionine hydroxy analogue (HMTBa-Ca), DL-Met protected with copolymer vinylpyridine/styrene (DL-Met-cop) and the isopropyl ester of the hydroxy analogue of methionine (HMTB-i) are currently authorised for use as feed additives, whereas DL-Met protected with ethylcellulose (DL-Met-ec) is not.

They all are applied for use in feedingstuffs. DL-Met, DL-Met-Na and HMTBa are also applied for use in water for drinking. DL-Met, DL-Met-Na, HMTBa and HMTB-Ca are intended for use in all animal species, the forms protected from ruminal degradation HMTB-i and DL-Met-cop for dairy cows and the DL-Met-ec for ruminants only.

2. Characterisation

The products are manufactured by chemical synthesis starting from methyl-thio-propionic aldehyde (MTPA) and hydrogen cyanide (HCN) in the presence of a catalyst (Kleemann et al., 1985). Purified MTPA and HCN are obtained by chemical synthesis using raw materials of petrochemical origin. The chemical reaction is normally complete and there is no raw material left.[10] Furthermore, any potential unreacted material will be eliminated. The manufacturing process is operated under the appropriate quality control system. Material Safety Data Sheets (MSDS) for all methionine forms are attached to the dossier.

2.1. DL-Methionine (DL-Met)

The additive is a solid product of white to yellowish colour containing by specification > 99 % DL-methionine (IUPAC name 2-amino-4-(methylthio)butanoic acid; CAS number 59-51-8). Its molecular weight is 149.2 g/mol, its molecular formula is $C_5H_{11}NO_2S$ and its molecular structure is given in Figure 1.

[10] Technical dossier/Section II/Annex II.3 01.

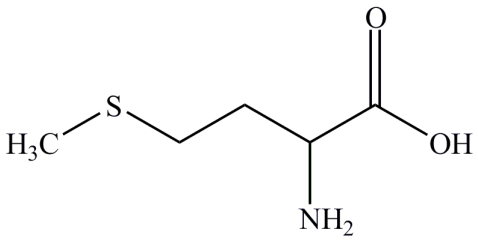

Figure 1. Molecular structure of methionine

Analysis of five samples of the additive from each producer (three) resulted in average contents of 99.4–99.7 % DL-Met (range of 15 samples 99.3–99.8 %). In all cases, the analytical data confirm the purity specification (> 99 %).

2.1.1. Impurities and physical properties of DL-Met

At least three batches per producer were analysed for impurities (As, Pb, Hg, Cd, dioxins and dioxin-like polychlorinated biphenyls (PCBs)).[11] The detected amounts of all these impurities were negligible in terms of the relevant EU legislation and were often below the detection limits. The mean particle size of DL-Met (three batches per producer) varied between 170 and 360 μm. The fraction below 50 μm amounted to 1–12 %, with no particles below 10 μm. Specific weight is 1.32–1.34 g/cm^3 and water solubility 2.9–3.4 g/100 ml (22–25 °C).

2.1.2. Stability and homogeneity of DL-Met

The applicant submitted data from three batches from one producer which asserted a shelf-life of 60 months when stored under cool and dry conditions (initial DL-Met content 99.7 %; final DL-Met content 99.7 %).

A vitamin mineral premixture containing 10 % DL-Met was stored for six months and no loss of DL-Met was observed. The applicant reported data from another premixture of unknown composition containing 20 % DL-Met. No decrease in DL-Met content could be detected after seven weeks at 50 °C or after an additional 27 weeks' storage at room temperature. Three other amino acid premixtures containing 20.0 %, 18.5 % and 15.6 % DL-Met were stored for seven months at room temperature and at 40 °C. Recovery rates did not fall below 93 %.

The stability of D-Met was studied in one batch of feed from one applicant. A broiler-type diet containing 0.2 % DL-Met was conditioned (at 65 °C and 90 °C) or expanded, pelleted and subsequently stored for three months. Feed processing and storage did not affect the DL-Met content.

To demonstrate the stability of DL-Met in water for drinking, water containing DL-Met at concentrations of 0.1 g/l, 0.5 g/l, 1.0 g/l, 5.0 g/l and 10.0 g/l was kept at room temperature (approximately 20–25 °C) for three days. Sampling was carried out daily, with one sample per concentration. No losses could be observed. On request, a second set of data, following the same experimental design as above, but at 22–24 °C, was provided for three batches of DL-Met. DL-Met was stable in water for drinking and no differences between the batches (one producer) could be observed.

To demonstrate homogeneity, the applicant provided details of a study of one producer in which different markers of a maize-soybean based broiler starter diet were compared at different mixing times.[12] The feed composition and incorporation rate of DL-Met were not provided. After five minutes of mixing, the DL-Met concentration in ten samples showed a coefficient of variation (CV) of 9.5 %.

[11] Supplementary information, August 2011, Annexes 1A–C, 2 and 3.
[12] Supplementary information, August 2011, Annex 4.

2.2. DL-Methionine sodium salt (DL-Met-Na)

The additive is an amber to light-brown clear liquid containing by specification a minimum of 40 % DL-Met and 6.2 % sodium and a maximum of 53.8 % water (IUPAC name 2-amino-4-(methylthio)butanoic acid sodium salt; CAS number 41863-30-3). Its molecular weight is 159.2 g/mol, its molecular formula is $(C_5H_{11}NO_2S)Na$ and its molecular structure is provided in Figure 2.

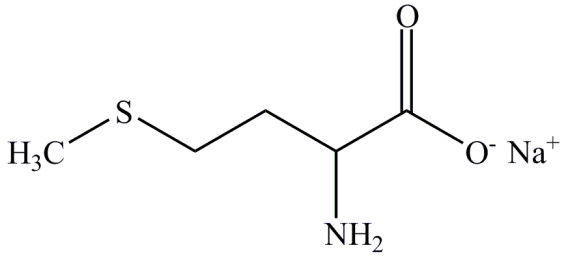

Figure 2. Molecular structure of methionine sodium salt

Analysis of five samples (from one producer) of the additive resulted in average content of 44.4 % DL-Met (range 44.3–44.4 %), 6.4 % sodium (range 6.3–6.4 %) and 53.3 % water (range 53.2–53.3 %).[13] The analytical data support the specification.

2.2.1. Impurities and physical properties of DL-Met-Na

Three batches were analysed for impurities (As, Pb, Hg, Cd, dioxins and dioxin-like PCBs).[14] The detected amounts of all these impurities were negligible in relation to the relevant EU legislation and were often below the detection limits.

The applicant reported that DL-Met-Na is completely dispersible in water. The pH value of the product is > 11 and its specific weight is 1.2 g/cm^3 (at 20 °C).

2.2.2. Stability and homogeneity of DL-Met-Na

The shelf-life of one batch was reported to be 24 months at both room temperature and 50 °C (initial DL-Met content 40.1 %; final DL-Met content 40.0 %).

It is envisaged that liquid DL-Met-Na will be incorporated not in premixtures but directly into final feed.

Data on stability in feed were not submitted. The applicant argued that methionine from DL-Met-Na is expected to be present in the final feed as a solid. Therefore, the applicant considers the stability figures submitted for the solid source of DL-Met to be valid also for the liquid source, DL-Met-Na. However, the applicant, under the heading of a homogeneity study, did provide data on the stability of methionine from DL-Met-Na in a typical broiler feed (composition given) stored for three days at temperatures of 45, 65 and 85 °C. The target concentration was 0.3 % supplemental methionine. Only at the highest temperature was any loss observed after three days, and this was minor (about 10 %).

To demonstrate the stability of DL-Met-Na in water for drinking, water containing DL-Met at concentrations of 0.1 g/l, 0.5 g/l, 1.0 g/l, 5.0 g/l and 10.0 g/l was kept at room temperature of approximately 20–25 °C for three days. Sampling was carried out daily, with two samples per concentration. No losses could be observed. On request, another batch was examined, following the same experimental design as above but at 22–24 °C. Sampling was carried out daily, with three samples per concentration. The data confirm the results described for the first batch.

[13] Technical dossier, section II.
[14] Supplementary information, August 2011, Annex 7.

The applicant did not submit data to demonstrate the capacity of liquid DL-Met-Na for homogeneous distribution in a final feed. In response to the EFSA's request, the applicant stated that comparable figures had already been provided for the hydroxy analogue of methionine, another liquid source of methionine, and that there is no reason to expect data for DL-Met-Na to be any different as the proper spreading and mixing is basically determined by the technical equipment used.

2.3. Hydroxy analogue of methionine (HMTBa)

The additive is an amber to dark amber liquid containing by specification a minimum of 88 % active substance (hydroxy analogue of methionine) and a maximum of 12 % water (IUPAC name 2-hydroxy-4-(methylthio)butanoic acid; CAS number 583-91-5). Its molecular weight is 150.2 g/mol, its molecular formula is $C_5H_{10}O_3S$ and its molecular structure is given in Figure 3.

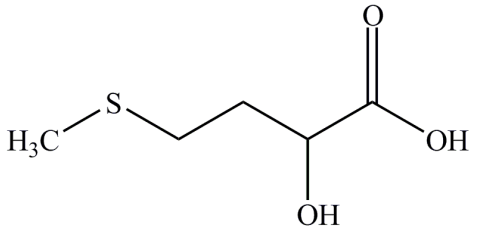

Figure 3. Molecular structure of the hydroxy analogue of methionine

Analysis of five samples of the additive from each of three producers resulted in average contents of 88.9–89.6 % active substance (range 88.8–89.7 %). The analytical data comply with the purity specification (> 88 %).

2.3.1. Impurities and physical properties of HMTBa

At least three batches per producer were analysed for impurities (As, Pb, Hg, Cd, dioxins and dioxin-like PCBs).[15] The detected amounts of all these impurities were negligible in relation to the relevant EU legislation and were often below the detection limits.

The applicant reported that HMTBa is completely dispersible in water. The pH value of the product is < 1 and its specific weight 1.21–1.23 g/cm^3 (at 20–25 °C).

2.3.2. Stability and homogeneity of HMTBa

The shelf-life of three batches of HMTBa was followed for 60 months under ambient conditions. No losses were observed (initial HMTBa content 89.6 %; final HMTBa content 89.3 %).

As HMTBa is a liquid, the additive will not be used in premixtures.

The additive was sprayed on a mixture of the other feed components (turkey finisher, composition given) at a concentration of 0.125 %. Samples were taken from the mash and the pelleted feed. Pelleted samples were also taken during storage for a total of 160 days. The concentration of the additive after pelleting (65 °C) was 0.105 %, compared with 0.122 % in the mash sample. No losses were observed during storage. An additional study was performed of the stability of HMTBa in three different feeds: a broiler finisher diet (inclusion rate of the additive 0.19 %), a turkey finisher diet (inclusion rate 0.13 %) and a layer finisher feed (inclusion rate 0.16 %). No further details were provided. The recovery in the three feeds, calculated as percentage of the inclusion rate, was 92 %, 95 % and 83 %, respectively, at the start of the study and 83 %, 93 % and 80 %, respectively, after three months.

[15] Supplementary information August 2011, Annexes 10–12.

The stability of HMTBa in water at room temperature (25–29 °C) was studied for four days in two samples of an aqueous dilution of a premixture containing HMTBa, formic acid and propionic acid, whereby the final concentration of HMTBa in water was 0.035 %. No losses were observed. The applicant noted that the premixture used to incorporate the HMTBa into water is currently the only real application of HMTBa in water for drinking. Stability data for two other batches of the additive in water for drinking were supplied on request. Intended concentrations of HMTBa in water were 0.025 %, 0.400 % and 1.000 %. Water was stored for 3 days at room temperature in ambient light at a laboratory workbench. No losses were observed. HMTBa is considered stable when added to water for drinking for at least three days.

Homogeneous distribution of the additive could be demonstrated from data reported in the stability study with the turkey finisher feed. The CV of eight samples each ranged between 2.7 % for the mash feed and 3.0 % for the pelleted feed.

2.4. Calcium salt of the hydroxy analogue of methionine (HMTBa-Ca)

The additive is a tan-coloured solid containing by specification a minimum of 84 % active substance, a minimum of 12 % calcium and a maximum of 1 % water (IUPAC name 2-hydroxy-4-(methylthio)butanoic acid, calcium salt; CAS number 4857-44-7). The molecular weight of the active substance is 338.45 g/mol, its molecular formula is $(C_5H_9O_3S)_2Ca$ and its molecular structure is shown in Figure 4.

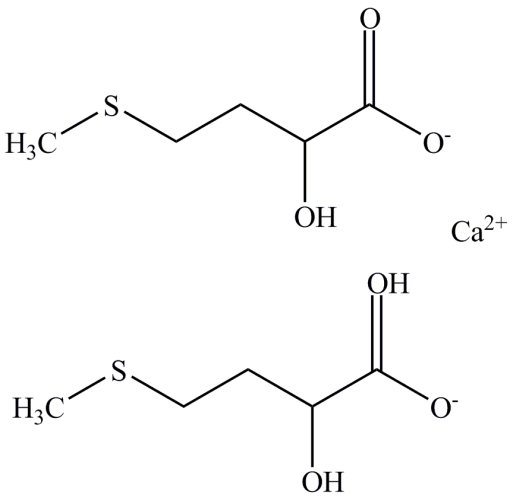

Figure 4. Structural formula of the calcium salt of the hydroxy analogue of methionine

Analysis of five samples of the additive (from one producer) resulted in average content of 85.4 % HMTBa (range 85.2–85.6 %), 12.9 % calcium (range 12.7–13.0 %) and 0.4 % water (range 0.28–0.50 %). The analytical data substantiate the purity specification (> 84 % HMTBa).[16]

2.4.1. Impurities and physical properties of HMTBa-Ca

Three batches were analysed for impurities (As, Pb, Hg, Cd, dioxins and dioxin-like PCBs).[17] The detected amounts of all impurities were negligible in relation to the relevant EU legislation and were often below the detection limits.

In five batches, most particles (97.5–100 %) showed diameters between 100 and 1400 μm. The fraction below 53 μm ranged from 0 % to 0.1 %.[18] Solubility in water is 7.4 g/100 ml (at 25 °C) and the specific weight is 0.7–0.8 g/cm³.

[16] Technical dossier, section II.
[17] Supplementary information, August 2011, Annexes 14A–C.
[18] Technical dossier, section II.

2.4.2. Stability and homogeneity of HMTBa-Ca

The shelf-life of five batches of HMTBa-Ca was followed for four years under ambient conditions. No losses were observed (initial HMTBa content 85.8 %; final HMTBa content 86.4 %).

The stability of HMTBa-Ca was examined in a mineral pig premixture (composition not given) with target supplementation of 4.4 % HMBTa for six months at room temperature.[19] Recovery expressed as percentage of the inclusion rate was 98 % at the start of the study, 102 % after three months and 86 % at the end of the study.

The stability of HMTBa-Ca in feed was analysed in two samples of each of two types of compound feed, a broiler starter and a broiler finisher diet; intended inclusion rates were 0.3 % and 0.27 % HMTBa, respectively.[20] Feed was stored for six months at room temperature. The initial HMTBa concentrations were not analysed. After one month, recoveries (as a percentage of target concentration of HMTBa) were 87 % and 68 % in the starter diet and 74 % and 86 % in the finisher diet. The corresponding data after six months were 89 % and 96 % and 99 % and 113 %, respectively.

To demonstrate the capacity to homogeneously distribute HMTBa-Ca in feed, ten samples of a standard mash feed with a target concentration of 0.12 % HMTBa-Ca were analysed and found to have mean value 0.135 % and a CV of 8.1 %.

2.5. Isopropyl ester of the hydroxy analogue of methionine (HMTB-i)

The additive is a light-brown liquid containing by specification a minimum of 95 % active substance and a maximum of 0.5 % water (IUPAC name isopropyl ester of 2-hydroxy-4-(methylthio)butanoic acid; CAS number 57296-04-5). Its molecular weight is 192.28 g/mol, its molecular formula is $C_8H_{16}O_3S$ and its molecular structure is given in Figure 5.

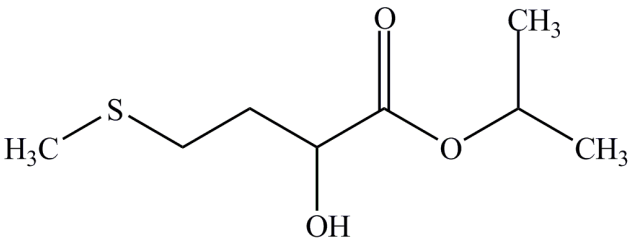

Figure 5. Molecular structure of the isopropyl ester of the hydroxy analogue of methionine

This product originates from one producer. The analysis of five samples of the additive resulted in an average content of 97.7 % HMTB-i (range 97.4–98.4 %) and 0.17 % water (range 0.11–0.25 %). The analytical data comply with the purity specification (> 95 %).[21]

2.5.1. Impurities and physical properties of HMTB-i

Three batches were analysed for impurities (As, Pb, Hg, Cd, dioxins and dioxin-like PCBs).[22] The detected amounts of all these impurities were negligible in relation to the relevant EU legislation and were often below the detection limits.

Water solubility was reported to be 25.1 g/l (at 30 °C); the pH of the additive is 3.6 and its specific weight 1.07 g/cm^3.

[19] Technical dossier, section II; supplementary information, August 2011.
[20] Technical dossier, section II; supplementary information, August 2011.
[21] Technical dossier, section II.
[22] Supplementary information, August 2011, Annexes 16A–C.

2.5.2. Stability and homogeneity of HMTB-i

Three batches of the additive were stored in glassware at 20–25 °C. The product was stable for three years (initial HMTB-i content 98.3 %; final HMTB-i content 97.1 %).

A first series of stability studies was performed in complementary feed for ruminants. A protein-rich feed (based on groundnuts, rape seed meal and soybean meal) was supplemented with 2.5 % or 1 % of the product (equivalent to 2.22 % and 0.9 % HMTB-i), pelleted and stored at ambient temperature for three months. Pelleting resulted in a loss of about 7–16 % compared with the original value. During storage, losses ranged between 5 % and 12 % in the case of the higher supplementation rate and between 10 % and 6 % for the lower supplementation rate.

A second series of 3-month stability studies was carried out on six different pelleted complementary feeds, one containing no cereals (control feed) and the others containing between 45 % and 50 % cereals (maize, barley, wheat, wheat by-products), five of them supplemented with 0.5 % HMTB-i and one supplemented with 3.0 % of the additive. The control feed remained stable over the observation period, whereas all of the other feeds lost about 12 %, 19 % and 22 % after 1, 2 and 3 months, respectively.

Three studies, each with a mash feed, with the addition of molasses and a pelleted feed and five samples of the nine different feeds, and target concentrations of 2.5 % (three feeds) and 1.0 % (six feeds) were submitted. It was shown that HMTB-i could be homogeneously incorporated in ruminant feed (CV 1.1–3.7 %).

2.6. DL-Methionine technically pure protected with copolymer vinylpyridine/styrene (DL-Met-cop)

The additive is a solid product, in the form of beadlets, containing by specification a minimum of 74 % DL-Met and a maximum of 19 % stearic acid, maximum 3 % copolymer poly(2-vinylpyridine) co-styrene, maximum 0.5 % ethylcellulose and sodium stearate.

Analysis of five samples of the additive (from one producer) revealed an average content of 77.7 % DL-methionine (range 77.3–78.2 %) and 2.1 % copolymer poly(2-vinylpyridine) co-styrene (range 1.97–2.24 %). The analytical data meet the purity specification (> 74 % DL-Met).[23]

2.6.1. Impurities and physical properties of DL-Met-cop

Three batches were analysed for impurities (As, Pb, Hg, Cd, dioxins and dioxin-like PCBs).[24] The detected amounts of all these impurities were negligible in relation to the relevant EU legislation and were often below the detection limits.

Most of the particles (> 99 %) of five batches were larger than 1400 µm.[25]The fraction of particles with a diameter smaller than 50 µm was below 1 % in all determinations.

2.6.2. Stability and homogeneity of DL-Met-cop

The applicant provided data showing the shelf-life of methionine in the additive in three batches over three years (initial methionine content 74.5 %; final methionine content 77.9 %) when stored in plastic cans, protected from light, at a temperature between 20 and 25 °C.

The applicant provided details of a further shelf-life study of three batches over three years, in which the integrity of protection could be demonstrated by measuring methionine release over 24 hours in a buffer solution at pH 6 and 40 °C, simulating the pH and temperature of rumen fluid. Under these

[23] Technical dossier, section II.
[24] Supplementary information, August 2011, Annexes 18A–C.
[25] Technical dossier, section II.

conditions, protection expressed as percentage of the non-released methionine was 96 % initially and 91 % at the end. The availability of methionine from DL-Met-cop was determined as the methionine release during two hours in a buffer solution at pH 2 and 40 °C. The liberation was 94 % initially and 104 % after three years.

Stability studies in complementary feed for ruminants were carried out following the same procedure. DL-Met-cop was incorporated in a mineral feed at a concentration of 4 % (20 % Ca, 5 % P and 5 % Mg). The samples were stored at room temperature for six months. Methionine protection (five samples per time point) was 92.9 % initially and 91.3 % at the end of the study. A crumbled protein concentrate (42 % CP; soybean meal, rapeseed meal, molasses, trace elements and vitamins) containing 1.5 % added DL-Met-cop was stored for six months at room temperature. The initial protection rate (one sample) was 96.1 % and the final rate 90.6 %. Another protein concentrate (40 % CP, with the same feed materials and the same level of added DL-Met-cop) in mash form showed a constant methionine protection rate (95 %) over five months.

Ten samples of a customer mineral mix with a target concentration of 11 % DL-Met-cop were analysed using the above-described methods. The CV was 8 %.

2.7. DL-Methionine technically pure protected with ethylcellulose (DL-Met-ec)

The additive is a white solid, in the form of cylindrical pellets, containing a minimum 85 % DL-Met, maximum 8 % starch, maximum 4 % ethylcellulose, maximum 1.5 % sodium aluminium silicate and maximum 1 % sodium stearate. The maximum water content is 2 %.

The product originates from one producer. Analysis of five batches of the additive resulted in an average content of 86.5 % DL-Met (range 86.2–86.8 %). The minimum specification for DL-Met (> 85 %) was exceeded in all samples.[26] The water content was constantly below (average 0.6 %) the specification.

2.7.1. Impurities and physical properties of DL-Met-ec

Three batches were analysed for impurities (As, Pb, Hg, Cd, dioxin and dioxin-like PCBs).[27] The detected amounts of all these impurities were negligible in relation to the relevant EU legislation and were often below the detection limits.

The applicant argues that the pelleted product is virtually dust free based on its high durability. Specific weight is 1.2 g/cm^3 and bulk density 65 kg/m^3.

2.7.2. Stability and homogeneity of DL-Met-ec

Methionine in DL-Met-ec remained stable for three years (initial methionine concentration 86.1 %; final methionine concentration 86.0 %) when stored under cool and dry conditions. The stability of the protected form, DL-Met-ec, was followed by analysing released methionine after shaking (frequency 120/min) in a water bath at 37 °C for 6 and 24 hours. The average proportion of protected methionine in the three batches amounted to 80 % after 6 hours and to about 40 % after 24 hours. After three years, the figures obtained were approximately the same.

The persistence of the coating during the mixing process (0, 2, 4, 6 and 8 min) was studied by testing samples from six different mineral feeds (10 % DL-Met-ec) during storage in molasses (40 % DL-Met-ec) for eight days or in six randomly selected types of silages (2 % DL-Met-ec) for two days. The so-called "protection rate" in all studies and under all conditions was > 99 %.

Stability data for premixtures and feedingstuffs as requested by Commission Regulation (EC) No 429/2008 were not submitted. Following the EFSA's request for such data, the applicant responded

[26] Technical dossier, section II.
[27] Supplementary information, August 2011, Annex 25.

that it would not be appropriate to provide stability data for all conceivable compound feed compositions, as the present dossier is a generic submission for the reauthorisation of a nutritional feed additive. The applicant further stated that DL-Met-ec has been marketed in Europe for ruminant feeding for more than 20 years without any complaint on stability or mixability. Thus, the applicant concludes that the product composition and protection are appropriate.

The homogeneous distribution of 2 % DL-Met-ec in soybean meal and a crumbled feed was examined after different mixing times. Eight to ten samples were analysed for each time point. After 2.5 min of mixing, a CV of 5.5 % was obtained in both feed types.

2.8. Conditions of use

The different additives under application are foreseen for use in feed or water for different animal species. Table 2 summarises the details of the proposed conditions of use.[28]

Table 2. Conditions of use of the different methionine sources

Additive	Animal species	Proposed administration via		
		Premixture	Final feed	Water for drinking
DL-Met	All	Yes	Yes	Yes
DL-Met-Na	All	No	Yes	Yes
DL-Met-cop	Dairy cows	Yes	Yes	No
DL-Met-ec	Ruminants	Yes	Yes	No
HMTBa	All	No	Yes	Yes
HMTBa-Ca	All	Yes	Yes	No
HMTB-i	Dairy cows	No	Yes	No

2.9. Evaluation of the analytical methods by the European Union Reference Laboratory (EURL)

EFSA has verified the EURL report as it relates to the methods used for the control of DL-Met, DL-Met-Na, HTMBa, HTMBa-Ca, HTMBa-i, DL-Met-cop and DL-Met-ec when used in animal nutrition. The Executive Summary of the EURL report can be found in Appendix A.

3. Safety

3.1. Safety for the target species

Tolerance studies of essential amino acids such as methionine and methionine analogues cannot be designed according to the protocols of conventional toxicity experiments because high dietary concentrations of a particular amino acid will result in amino acid imbalances with depression of feed intake and hence impaired performance. Consequently, tolerance studies are not required for amino acids naturally occurring in the proteins of animals or plants already authorised as feed additives (Regulation (EC) No 429/2008). However, Ball et al. (2006) proposed the use of requirement experiments to identify tolerable upper intakes of amino acids, particularly in humans.

3.1.1. Methionine and its analogue

Methionine and its analogues are the most toxic amino acids. Thus, addition of 4 % DL-Met to a maize soybean diet reduced weight gain in chickens for fattening by 92 % and feed intake by about 56 % (Edmonds and Baker, 1987a). In a dose–response study in chickens fed a purified diet (0.35 % L-Met, 0.35 % L-cystine), the addition of 0.5–2.0 % L-Met resulted in a linear reduction in weight gain of 11–

[28] Supplementary information, August 2011.

67 % and in feed efficiency of 7–37 % (Harter and Baker, 1978). Comparable results were obtained in piglets fed 2 % or 4 % additional DL-Met in a maize/soybean-based diet: weight gain was reduced by 12 % and 40–52 % and feed intake by 17 % and 39–44 %, respectively (Edmonds and Baker, 1987b; Edmonds et al., 1987).

Studies of chickens fed a basal diet (0.35 % DL-Met, 0.35 % L-Cys) containing added isosulphurous L-methionine (1.75 %) or L-, D- or DL-HMTBa (2.00 %) have shown that the toxicity of L-Met and D-HMTBa is similar, with DL-HMTBa being of moderate toxicity and L-HMTBa being least toxic when assessed according to weight gain, feed efficiency and spleen iron concentration (Baker and Boebel, 1980). These results are in line with the observation that the D-isomer of HMTBa is biologically more active than the L-isomer (Baker and Boebel, 1980; Baker 1986). The lower toxicity of HMTBa compared with DL-Met has been also confirmed in Pekin ducks (Xie et al., 2007). There obviously exists an interaction between HMTBa and cyst(e)ine, as even small excesses of cyst(e)ine appear to depress feed intake and weight gain and reduce HMTBa utilisation compared with DL-Met (Katz and Baker, 1975; Baker and Boebel, 1980; Baker, 2007; Dilger and Baker 2008).

It appears that the toxicity of methionine and its analogues can be attributed to transamination processes, leading to the formation of the intermediate 3-methylthiopropionate, and subsequently to the volatile sulphur compounds methanethiol and/or hydrogen sulphide (Mitchell and Benevenga, 1978; Steel and Benevenga1978, 1979; Steel et al., 1979; Finkelstein and Benevenga, 1984, 1986). The key role of the transamination pathway in methionine toxicity has recently been confirmed (Dever and Elfarra, 2008)

3.1.2. Copolymer 2-vinylpyridine/styrene

The copolymer 2-vinylpyridine/styrene has been the subject of *in vivo* studies in the target animals.[29] The studies tested the product administered both as the coating only and in the form of a protected amino acid. A single dose of copolymer (0, 6.3, 63, 126, 315 or 630 mg per kg body weight) was administered to ten cattle aged 1 year and weighing 210–27 kg. No toxic effects (mortality or specific symptoms) were observed and there were no histological changes in any of the major organs examined. The copolymer proved to be almost completely non-toxic under the experimental conditions used.

In another study, the copolymer at doses equivalent to 0, 6.3, 18.9, 31.5 and 63 mg per kg body weight, in the form of a coating containing 31 % copolymer and (with the exception of the 63 mg/kg dose) or a complete protected amino acid formulation, was administered for 90 days to 40 Holstein steers weighing 200–350 kg and divided into groups of five animals. All doses were well tolerated. Clinical examination, haematological and biochemical tests and histopathological examinations did not detect any treatment-related effect.

3.1.3. Conclusions on the safety for species

Supplementing appropriate amounts of methionine and methionine analogues to meet requirements is safe for the target species. The earliest signs of a critical overdose are depressed feed intake and weight gain. The toxic levels of supplemented methionine and its analogues vary and depend on the basal diet and its content of sulphur-containing amino acids.

The supplementation of feedingstuffs with combined HMTBa and cysteine/cystine should be avoided as small excesses of cyst(e)ine appear to reduce HMTBa utilisation.

The copolymer 2-vinylpyridine/styrene is safe for ruminants when administered at use levels of the DL-Met-cop.

[29] Supplementary information, August 2011, Annex 21.

3.2. Safety for the consumer

As a general principle, conventional toxicology studies are considered to be inappropriate for testing pure substances which are dietary nutrients (essential dietary nutrients). In the case of such substances, there is a physiological concentration that is optimum for health and performance.

Dietary intakes of such substances in quantities that lead to body levels that are significantly below or above that which is optimum for health and performance will inevitably cause a physiological imbalance and consequent adverse effects. This principle applies to substances whose purity is well established and the source and method of production of which are sufficiently well characterised to provide reassurance that no toxic contaminants will be present in the product. The testing appropriate to such substances will need to be judged on a case-by-case basis but, provided the use of the substance leads to no increase in human intake, there is no requirement for further toxicity data.

Appendix B contains information on the absorption, distribution, metabolism and excretion of methionine and its analogue.

Methionine is mainly used for protein synthesis or is metabolised to homocysteine via *S*-adenosyl-methionine and *S*-adenosyl-homocysteine or transaminated to 3-methylthiopropionate and then to volatile sulphur compounds (methanethiol and/or hydrogen sulphide). Homocysteine can further be converted to cystathione and then to cysteine or recycled to methionine using 5-methyltetrahydrofolate or betaine (from diet or choline oxidation) as methyl donor.

3.2.1. Styrene and 2-vinylpyridine in DL-Met-co

The carry-over of styrene and 2-vinylpyridine from feed to milk has been studied in lactating cows.[30] In the first study, two groups of four cows were administered ^{14}C-styrene (labelled on the benzene ring) or ^{14}C-2-vinylpyiridine (labelled on the terminal carbon of the side-chain), each at a dose of 2 mg/kg body weight orally for 14 days. The low specific activity of the label necessitated the use of a dose that is considerably higher (about 10^6-fold) than the expected exposure of the animals to styrene resulting from the use of the protected amino acid, the consequence being an eventual underestimation of the absorption rate and percentage of carry-over to milk. Samples of urine, faeces, milk and blood were taken for measurement of total radioactivity. Absorption was found to be very rapid. The peak concentration in blood was observed after 1.43 (^{14}C-styrene) and 2.15 hours (^{14}C-2-vinylpyiridine). The major elimination route was the faeces (77 % and 73 %, respectively), followed by the urine (2.3 to 9.0 %). Passage in milk represented only 0.09 % and 0.89 % of the administered dose, respectively. Another study reinvestigated the fate of ^{14}C-2-vinylpyridine labelled on the pyridine ring, which is not subject to decarboxylation, and followed the same experimental protocol. Radioactivity excreted in milk represented only 0.34 % of the administered dose.

No data are available concerning the metabolic fate of styrene and 2-vinylpyridine in cattle. In humans and rodents, the first common step in the metabolic pathways of styrene is oxidation (cytochrome P450 dependent) to styrene-7,8-epoxide. Subsequently, styrene-7,8-epoxide is either hydrolysed by an epoxide hydrolase into phenylglycol and finally converted into mandelic acid and phenylglyoxylic acid excreted in the urine (in humans) or conjugated with glutathione to form mercapturic acids (in rodents, but also in humans at high styrene doses). As the key enzymes involved in the oxidation, hydrolysis and conjugation of styrene are also expressed in cattle, it is likely that styrene would follow one of these metabolic routes in this species. Moreover, styrene is actively metabolised by bacteria and therefore the ruminal flora should play a role in styrene metabolism in ruminants. No metabolic studies of 2-vinylpyridine in mammals have been published.

In order to evaluate consumer exposure to monomers that would result from the consumption of milk, the applicant made the following assumptions: (i) protected amino acids will be administered to cows at the highest recommended dose (150 g/cow/day); (ii) the co-polymer will be used at a dose of 3 %

[30] Supplementary information, August 2011, Annex 21.

and will contain the highest doses of monomers allowed by the specification (200 µg styrene and 200 µg 2-vinylpyridine per kg copolymer); and (iii) the total radioactivity measured in the milk will represent 0.09 % of the administered dose of styrene and 0.34 % of that of 2-vinylpyridine. The calculations on this basis indicated that (i) 0.9 µg of styrene and 0.9 µg of 2-vinylpyridine would be consumed per day by the cow, (ii) for a cow producing 5 kg milk per day (most concentrated output), the residues of both monomers would be 0.0016 µg equivalent styrene and 0.0061 µg equivalent 2-vinylpyridine/kg milk. These values are about three orders of magnitude lower than the levels of styrene found in a wide range of foodstuffs (Steele et al. 1994; Tawfik and Huyghebaert, 1998; MAFF, 1999; FDA, 2000).

No data regarding 2-vinylpyridine residues in food are available.

The FEEDAP Panel analysed the calculations and figures provided by the applicant and found the scenario acceptable. Based on consumption of 1.5 litres of milk per day, the exposure of consumers would amount to 0.0024 µg of styrene and 0.0092 µg of 2-vinylpyridine per day. For comparison, the Office of Food Additive Safety of the US Food and Drug Administration (FDA) established a cumulative estimated daily intake of 0.15 µg of styrene per kg body weight per day (corresponding to 9 µg per person per day) resulting from the transfer of styrene from packaging to food (FDA, 2011). This figure would increase by only 0.026 % if 1.5 litres of milk from DL-Met-cop-treated cows is consumed in addition.[31]

3.2.2. Conclusions

Methionine is incorporated in the protein of tissues and products, showing a constant amino acid pattern. Consequently, the use of the additives reported here will not result in an increased content of tissues and products. Doses exceeding the requirement will be excreted.

The FEEDAP Panel concludes that the use of DL-Met-cop in the nutrition of dairy cows would not raise concerns to consumer safety, since the residues present in the animal tissues and products resulting from the extremely small quantities from free styrene and 2-vinylpyridine are very unlikely to raise concerns on consumer safety.

3.3. Safety for the user

3.3.1. DL-Methionine

As more than 1 % of particles of this product are < 50 µm in diameter, an inhalation study was conducted according to OECD Guideline 403 and EC method B.2 (1992).[32] A group of five rats of each sex was exposed nose-only to an atmosphere containing DL-Met at 5.25 mg per litre of air for 4 hours. No mortality or signs of toxicity were observed and the animals gained the expected weight during the 14 days after exposure. Based on these results, DL-Met does not require any labelling regarding inhalation toxicity.

Eye irritation potential was investigated in three male rabbits according to OECD guideline 405 and EC method B.5.[33] Conjunctival redness was seen in all animals immediately after instillation but no effects were visible at subsequent observation times. It is concluded that DL-Met is non-irritant according to this procedure.

[31] At the moment this opinion was adopted, the Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) Panel was in the process of assessing the safety of styrene when used as flavouring food additive.
[32] Technical dossier, section III, reference III.301.
[33] Technical dossier, section III, reference III.3.2.

Skin irritation potential was investigated in three male rabbits according to OECD guideline 404 and EC method B.4.[34] No effects were seen following exposure; thus, it is concluded that DL-Met is non-irritant according to this procedure.

Skin sensitisation potential was assessed by a Buehler test in guinea pigs (OECD guideline 406; EC method B.6).[35] There was no evidence of any sensitisation potential in this study.

3.3.2. DL-Methionine sodium salt

As this is a liquid product, no investigation of inhalation toxicity is required. However, as the pH of this compound is > 11, it should be considered as corrosive to skin and eyes, and harmful if swallowed.

3.3.3. Hydroxy analogue of methionine

As this is a liquid product no investigation of inhalation toxicity is required.

Eye irritation potential was investigated in one study using six rabbits and another using two rabbits, neither study being compliant with current OECD guidelines or EC methods.[36] Severe effects seen in all animals in both studies resulted in the classification of a severe irritant in the first, and corrosive in the second one.

Skin irritation potential was investigated in four studies in rabbits (only three of which complied with OECD guideline 404.[37]) The fourth study is not considered further.[38] One study resulted in oedema and erythema, which took more than 72 hours to reverse. Another showed only erythema but still persisting for more than 72 hours. The third study showed very few effects 24 hours after treatment. Overall, the results are consistent with a classification of irritating to the skin.

Skin sensitisation potential was investigated in a human trial using more than 100 volunteers.[39] This study revealed a significant level of irritation of a 10 % solution, necessitating use of a 1 % solution in the induction and challenge phases. The study revealed no evidence of any sensitising potential in the panel of volunteers exposed.

3.3.4. Calcium salt of the hydroxy analogue of methionine

As less than 1 % of particles are < 50 μm in diameter, no investigation of inhalation toxicity is required.

Eye irritation potential was investigated in six rabbits using a protocol that preceded OECD guidelines.[40] Effects were confined to the conjunctiva, and severe erythema was recorded at all time-points. The product is considered irritant to eyes.

Skin irritation potential was investigated in six rabbits using a protocol that preceded OECD guidelines.[41] Any effects seen were extremely mild and limited to the first 48 hours following exposure. It is concluded that the product is not a skin irritant under the conditions of the study.

Skin sensitisation was not investigated for this product.

3.3.5. Isopropyl ester of the hydroxy analogue of methionine

As this is a liquid product, no investigation of inhalation toxicity is required.

[34] Technical dossier, section III, reference III.3.3.
[35] Technical dossier, section III, reference III.3.4.
[36] Technical dossier, section III, reference III.3.5.
[37] Technical dossier, section III, references III.3.7, 3.8 and 3.9.
[38] Technical dossier, section III, reference III.3.5.
[39] Technical dossier, section III, reference III.3.10.
[40] Technical dossier, section III, reference III.3.11.
[41] Technical dossier, section III, reference III.3.11.

Eye irritation was investigated in three rabbits according to OECD guideline 405 and EC method B.5.[42] The product caused effects primarily in the conjunctiva, but limited effects in cornea and iris were also reported. All effects were significantly reduced by 48 hours and cleared by five days after treatment. Based upon these results, the product does need to be labelled as an irritant under EU rules.

Skin irritation potential was investigated in three rabbits according to OECD guideline 404 and EC method B.4.[43] No effects were seen following exposure; thus, it is concluded that the product is non-irritant according to this procedure.

Skin sensitisation potential was assessed in a Magnusson & Kligman test in guinea pigs (OECD guideline 406; EC method B.6[44]). There was limited evidence of a response to challenge (one animal) and under the test protocol the results are interpreted to provide no evidence of dermal sensitisation potential.

3.3.6. DL-Methionine technically pure protected with copolymer vinylpyridine/styrene

As less than 1 % of particles are < 50 μm, no investigation of inhalation toxicity is required.

No data are provided for this product relating to irritancy or sensitisation.

3.3.7. DL-Methionine technically pure protected with ethylcellulose

As this product is pelleted and virtually dust-free, no investigation of inhalation toxicity is required.

No data are provided for this product relating to irritancy or sensitisation, but the properties of methionine alone are likely to be relevant.

3.3.8. Conclusions on safety for the user

None of the products are considered to present a significant inhalation risk.

DL-Met, DL-Met-cop and DL-Met-ec are considered non-irritant to skin and eyes, and the lack of sensitisation potential demonstrated for DL-Met is considered also relevant to DL-Met-cop and DL-Met-ec. DL-Met-Na is considered corrosive to skin and eyes and harmful if swallowed.

HMTBa is irritant to the skin and corrosive to the eyes but is not a dermal sensitiser. HMTBa-Ca is irritant to the eyes but not to the skin. The absence of a dermal sensitisation potential demonstrated for HMTBa is considered also relevant for HMTBa-Ca. HMTB-i is non irritant to the skin and eyes, and it is not a dermal sensitiser.

3.4. Safety for the environment

Methionine is a physiological and natural component in animals and plants. Like its salts and the hydroxy analogue, it is not excreted as such (but as urea/uric acid, sulphate and CO_2). The use of methionine, its analogue and their salts in animal nutrition would not lead to any localised increase in the concentration in the environment. It is concluded that the use of these products as feed additives does not represent a risk to the environment.

4. Efficacy

The efficacy of DL-Met and of the HMTBa in monogastric animals and fish is described in numerous publications. Their salts, DL-Met-Na and HMTBa-Ca, are not expected to behave differently. Normally, none of them requires any further demonstration of efficacy. However, the methionine enantiomers and the hydroxy analogues may show different bioefficacy.

[42] Technical dossier, section III, reference III.3.12.
[43] Technical dossier, section III, reference III.3.13.
[44] Technical dossier, section III, reference III.3.14.

4.1. Comparative bioefficacy of the methionine enantiomers and the hydroxy analogue

There remains controversy regarding the digestibility of HMTBa, and particularly the effectiveness of the HMTBa utilisation, in different animal species. This may be attributable to differences in experimental design and response criteria, the incomplete conversion of HMTBa to L-Met (Ferjancic-Biagini et al., 1995) and species differences in HMTBa utilisation (see Baker, 2006). Moreover, Baker and co-workers have identified different dietary factors that may interfere with the utilisation of HMTBa; even small excesses of cyst(e)ine appear to reduce HMTBa utilisation, and differences in the utilisation of HMTBa and DL-Met (on a equimolar or isosulphurous basis) are more pronounced when crystalline amino acid diets are fed rather than diets containing intact protein (Katz and Baker 1975; Baker and Boebel, 1980; Christensen et al., 1980a, b; Dilger and Baker, 2007, 2008).

Estimates of the bioequivalence of HMTBa and DL-Met in pigs have ranged from 73 % on an equimolar basis (64 % on weight basis, 88 % HMTBa), when nitrogen retention was used as response criterion (Kim et al., 2006), to about 100 % (equimolar) in another study in which weight gain was assessed (Knight et al., 1998). In laying hens, HMTBa bioequivalence was found to be about 88 % on a molar basis (Liu et al., 2004). In broiler chickens exposed to heat stress, the bioefficacy of HMTBa (equimolar) ranged from 67 % (feed conversion) to 83 % (weight gain) relative to DL-Met (Rostagno and Barbosa, 1995). However, other studies in chickens for fattening and taking a statistical approach indicate that the bioefficacy of HMTBa may be superior to that of DL-Met at levels used commercially, but inferior when dietary levels are deficient or as an overall conclusion from the multiple regression that the bioefficacy is equal (Vázquez-Añón et al., 2006a,b). It should be mentioned that the studies used for the statistical analysis are predominantly not accessible or have been published in non-peer-reviewed journals. The equimolar addition of HMTBa-Ca salt, HMTBa and HMTBa polymers to purified diets devoid of sulphur-containing amino acids resulted in bioefficacy values relative to DL-Met of 87 %, 78 % and 69 %, respectively, and supplementation of methionine-deficient semipurified feather meal diets for chicks resulted in bioefficacy values of 84 %, 77 % and 54 %, respectively (Boebel and Baker, 1982b). The authors explained the low bioefficacy of HMTBa and HMTBa-Ca in feather meal by the extreme deficiency of DL-Met combined with excesses of cystine, whereas the inferior efficacy of HMTBa polymer can be attributed to the lower DL-Met activity per se.

Growth and feed efficiency data indicate that channel catfish (*Ictalurus punctatus*) can utilise DL-Met as effectively as L-Met; however, the efficiency of HMTBa in promoting growth was only about 26 % of that of L-Met (Robinson et al., 1978).

The utilisation of various methionine analogues in different species is summarised in Table 3.

Table 3. Relative utilization of methionine isomers and its OH analogue[(1)] (Baker, 2006)

Amino acid	Rat	Mouse	Pig	Chick	Dog	Human
L-Met	*100*	*100*	*100*	*100*	*100*	*100*
D-Met	90	75	100	90	100[(2)]	30[(3)]
DL-Met	95	88	100	95	100	65
DL-OH-Met	70	70	80	80	NA	NA

DL-OH-Met, hydroxy analogue of methionine (HMTBa); NA, data unclear or not available.

(1) Values are expressed as percentages of the growth efficacy (molar or isosulphurous basis) of the L-isomer, which in all cases is presumed to represent 100 % oral utilisation.

(2) Efficacy of D-Met is also almost 100 % in growing kittens.

(3) Efficacy is also about 30 % in non-human primates.

4.2. Methionine and its hydroxy analogues for ruminants

Methionine and its precursor hydroxy analogue (as other amino acids), when fed to ruminants, are rapidly degraded by the ruminal microflora and therefore are largely not absorbed unchanged (Patterson and Kung, 1988; Noftsger et al. 2005). Consequently, they cannot contribute to the improvement of the quality of dietary protein. They need ruminal protection to become as efficacious as in monogastric animals. However, data from Volden et al. (1998) would indicate that a small amount (about 20 %) of methionine can escape rumen fermentation.

It should be considered if and to what extent the three methionine additives intended to be used in ruminants escape rumen fermentation and are absorbed in the gastrointestinal tract. The assessment does not contain any conclusion whether and under which conditions supplementation of ruminant feedingstuffs would result in better performance of animals fed with these protected forms.

4.2.1. Isopropyl ester of methionine hydroxy analogue (HMBT-i)

SCAN (2003) concluded that a proportion of the HMTB-i ingested with feed (possibly around 50 %) is available (as 2-hydroxy-4-(methylthio)butanoic acid (HMTBa)) to dairy cattle and can serve as a precursor of methionine.

Graulet et al. (2005) studied the plasma kinetics of HMTB-i in rumen-cannulated cows after administration of a spot dose (50 g methionine equivalent) to the liquid phase of the rumen content. Plasma concentrations of HMTB-i and its metabolites, HMTBa, methionine, isopropyl alcohol and acetone, were analysed. A calibration curve established by modelling the area under the curve response to increasing doses served as the main end point. It could be shown that HMBi was quickly absorbed and hydrolysed to HMTBa and isopropyl alcohol, and then converted to methionine and acetone. The availability of methionine from HMTB-i was estimated from different experimental runs to be about 50 %.

Rulquin et al. (2006) compared among others the plasma response of 16 cows to 21.3 g/day HMTB-i and 26.4 g/day HMTBa, on top of their usual ration, administered in two equal amounts. The plasma concentration of methionine (in a pooled sample collected 30 min before and 2 and 6 hours after morning feeding) was 34.7 µmol/l in the HMTB-i group compared with 16.5 µmol/l in the non-supplemented control group. Feeding HMTBa resulted in no change in plasma methionine.

4.2.2. DL-Methionine protected with coploymer vinylpyridine/styrene (DL-Met-cop)

The applicant provided on request three publications in support of ruminal protection and intestinal availability of DL-Met-cop. Robert et al. (1989) studied the effect of rumen-protected amino acids (daily doses calculated to be equivalent to 8 g of methionine and 24 g of lysine post-ruminally available) at two levels of protein (152 and 168 g CP per kg dry matter) on the plasma level of amino acids in four double-cannulated dairy cows. Plasma methionine (measured 10 days after study start) increased from 0.17 to 0.33 mg/100 ml in cows fed the low-protein diet and from 0.18 to 0.35 mg/100 ml in cows fed the high-protein diet as a result of methionine supplementation. Plasma lysine increased correspondingly.

Two other studies of encapsulated rumen-protected methionine and lysine in dairy cows were published by Rogers et al. (1987, 1989). In the first study, in a total of 18 cows, the authors supplemented a blended diet consisting of corn silage and corn and soybean meal with varying amounts of (lysine and) methionine (0, 4.52, 10.4 or 16.28 g/day) for three weeks. Blood samples were taken on days 19 and 21. Plasma methionine increased linearly ($P < 0.01$) with methionine concentration in the coated source (0.25 mg/100 ml in the control group, 0.36 and 0.33 mg/100 ml in the low-methionine group, 0.37 mg/100 ml in the intermediate-dose group and 0.61 and 0.53 mg/100 ml with the high-methionine group).

In the second study, in a 3 × 3 factorial design, the effects of rumen-protected methionine (and lysine) were studied in a total of 130 dairy cows from three universities. The cows were individually fed a diet consisting of corn silage and a corn grain-based supplement with soybean meal or corn gluten plus urea. Methionine at a dose of 3.4, 10.4 or 12.2 g/day (estimated amounts supplied post-ruminally) was administered for 283 days, along with three different lysine doses (5.9, 13.5 and 21.1 g/day). Blood samples were collected once during 41 to 59 d of lactation. Plasma concentrations increased linearly with supplementation of rumen-protected methionine (and lysine) when the soybean meal-containing supplement was given ($P < 0.01$).

These results can be considered as indicative for rumen protection and intestinal availability of the coated methionine tested. However, none of the publications contains a more detailed description of the protected methionine (and lysine). Product identity to the DL-Met-cop under application is not demonstrated.

4.2.3. DL-Methionine technically pure protected with ethylcellulose (DL-Met-ec)

Overton et al. (1996) studied the release of DL-Met-ec in three cows fitted with ruminal and duodenal cannulas by inserting polyester bags containing 0.75 g of DL-Met-ec (85.1 % DL-Met) for 3, 6, 12, 24 and 96 hours. The methionine content of the DL-Met-ec and residues remaining in the polyester bags after exposure to ruminal fermentation and after collection of bags from the faeces was used to calculate the extent to which methionine was protected from ruminal fermentation and the extent to which it was released into the total tract. Ruminal disappearance of methionine averaged 5.8 %, 8.1 %, 21.8 %, 37.5 % and 87.5 % after 3**,** 6, 12, 24, and 96 hours of incubation, respectively. Post-rumina1 disappearance of methionine from bags inserted in the duodenum after treatment with pepsin hydrochloride averaged 63.4 %, 62.6 %, 51.6 %, 43.6 % and 8.8% for bags incubated in the rumen for 3, 6, 12, 24 and 96 hours, respectively.

DL-Met-ec appeared to be effectively protected against degradation within the rumen if residence time in the rumen was ≤ 6 hours; however, longer residence time in the rumen resulted in greater disappearance of methionine from the bags. Although substantially more methionine disappeared from the polyester bags into the rumen as residence time in the rumen increased beyond 6 hours, a larger proportion of the methionine was also released into the total tract. These data suggest that DL-Met-ec supplied methionine post-ruminally to lactating dairy cows in this experiment.

Berthiaume et al. (2000) compared the intestinal disappearance of DL-Met-ec determined *in situ* or *in vivo* over three consecutive periods. Four non-lactating Holstein heifers with cannulae in the rumen, duodenum and ileum were used. A total of 16 bags, each containing 1.5 g of ruminally protected methionine, were incubated in the rumen (4.5 hours) of each cow and then transferred to an acid-pepsin solution to simulate the abomasum (2.5 hours). Following incubation, the bags were introduced directly into the duodenal or ileal cannula for the *in situ* method, while for the *in vivo* method the content of remaining bags was transferred into gelatin capsules before their introduction in the duodenal or ileal cannula. The disappearance of methionine in the small intestine *in vivo* tended to be higher than *in situ* (74.5 % vs. 43.7 %). The results indicated that when used to assess intestinal availability of ruminally protected methionine, the mobile nylon bag technique can underestimate the true bioavailability of methionine. They also demonstrate the intestinal availability of methionine from ruminally preincubated DL-Met-ec.

Berthiaume et al. (2001) conducted another study to compare the rates of disappearance of amino acids from the small intestine and their net appearance in the blood draining the small intestine or gastrointestinal tract in five cows (two equipped with duodenal and ileal cannulae, three with a duodenal cannula only). The cows were fed a total mixed ratio top dressed with 0 or 72 g/day DL-Met-ec. The addition of ruminally protected methionine to the diet increased the duodenal flux of methionine, leading to a higher apparent digestibility of methionine in the small intestine; 66 % of methionine from DL-Met-ec bypassed the rumen, and 82 % of that methionine disappeared from the

small intestine. Arterial plasma methionine concentrations numerically increased with DL-Met-ec, while total amino acid concentration decreased.

The studies indicate that methionine from DL-Met-ec escapes rumen fermentation to a certain extent and that the amount reaching the small intestine is available. About half of the methionine from DL-Met-ec appears to be available for protein synthesis.

4.3. Conclusions

DL-Met and its sodium salt as well as HMTBa and its calcium salt are effective dietary sources of methionine for protein synthesis in monogastric animals (and fish). The hydroxy analogues show a somewhat lower bioefficacy than the DL-Met forms. They could also be used in ruminants, particularly when these active substances are used in specially formulated additives or complementary feedingstuffs to protect them against degradation by the ruminal microbiota.

The coated and otherwise protected methionine forms, DL-Met-cop, HMTB-i and DL-Met-ec, escape ruminal degradation to a certain extent, the remainder being available for gastrointestinal absorption. However, product identity of the DL-Met-cop used in studies with the product under application was not demonstrated.

5. POST-MARKET MONITORING

The FEEDAP Panel considers that there is no need for specific requirements for a post-market monitoring plan other than those established in the Feed Hygiene Regulation[45] and Good Manufacturing Practice.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

Supplementing appropriate amounts of methionine and methionine analogues to meet requirements is safe for the target species. The earliest signs of a critical overdose are depressed feed intake and weight gain. The toxic levels of supplemented methionine and its analogues vary and further depend on the basal diet and its content of sulphur amino acids.

Methionine from DL-Met, its hydroxy analogue and their salts is incorporated in the protein of tissues and products, showing a constant amino acid pattern. Consequently, the use of these additives will not result in an increased content in tissues and products. Doses exceeding the requirement will be excreted.

Residues present in the animal tissues and products resulting from the extremely small quantities from free styrene and 2-vinylpyridine found in DL-Met-cop are very unlikely to raise concerns on consumer safety.

None of the products are considered to present a significant inhalation risk. DL-Met, DL-Met-cop and DL-Met-ec are considered non-irritant to skin and eyes, and the lack of sensitisation potential demonstrated for DL-Met is considered also relevant to DL-Met-cop and DL-Met-ec. DL-Met-Na is considered corrosive to skin and eyes and harmful if swallowed. HMTBa is an irritant to the skin and corrosive to the eyes but is not a dermal sensitiser. HMTBa-Ca is irritant to the eyes but not to the skin. The absence of a dermal sensitisation potential demonstrated for HMTBa is considered also relevant for HMTBa-Ca. HMTB-i is non-irritant to the skin and eyes, and it is not a dermal sensitiser.

The use of these products as feed additives does not represent a risk to the environment.

[45] Regulation (EC) No 183/2005 of the European Parliament and of the Council of 12 January 2005 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

DL-Met and its sodium salt as well as HMTBa and its calcium salt are effective dietary sources of methionine for protein synthesis in monogastric animals (including fish). The hydroxy analogues show a somewhat lower bioefficacy than the DL-Met forms. They can all be used in ruminants, particularly when they are included in specially formulated additives or complementary feedingstuffs to protect them against degradation by the ruminal microbiota. The coated and otherwise protected methionine forms, DL-Met-cop, HMTB-i and DL-Met-ec, escape ruminal degradation to a certain extent, the remaining is available for gastrointestinal absorption. However, product identity of the DL-Met-cop used in studies with the product under application was not demonstrated.

RECOMMENDATIONS

Complete diets for all animal species, and particularly those for food-producing animals, contain well-balanced protein by using amino acid supplementation optimally adjusted to the specific requirements of the different animal species and categories for essential amino acids (the ideal protein concept). Each additional administration of individual essential amino acids will negatively affect the amino acid balance, and consequently the performance of animals, as well as target animal safety in a dose-dependent manner. Because of these concerns, the FEEDAP Panel strongly recommends that the use of methionine (all substances under application) is not authorised in water for drinking.

The combined supplementation of feedingstuffs with HMTBa and cysteine/cystine should be avoided as small excesses of cyst(e)ine appear to reduce HMTBa utilisation.

The conditions of use of the ruminal protected additives, as proposed by the applicant, differentiate between the use in dairy cows (HMTB-i and DL-Met-cop) and ruminants (DL-Met-ec). As ruminal protection is a principal issue, the FEEDAP recommends the use of all protected forms to be foreseen in ruminants.

DOCUMENTATION PROVIDED TO EFSA

Methionine, its salts and analogues. April 2010. Submitted by AMAC EEIG.

Methionine, its salts and analogues. Supplementary information. August 2011. Submitted by AMAC EEIG.

Methionine, its salts and analogues. Supplementary information addendum. January 2012. Submitted by AMAC EEIG.

Evaluation report of the Community Reference Laboratory for Feed Additives on the methods(s) of analysis for methionine

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APPENDIX A

Executive Summary of the Evaluation Report of the Community Reference Laboratory for Feed Additives on the Method(s) of Analysis for Methionine

In the current application authorisation is sought for seven forms of *methionine* under Articles 4(1) and 10(2), under the category/functional group 3(c) 'nutritional additives'/'amino acids, their salts and analogues', according to Annex I of Regulation (EC) No 1831/2003.
According to the Applicant:

- *DL-methionine* (*DLM*) is intended for all animal species and categories with a minimum purity of 99%;
- *liquid sodium DL-methionine* (*DLM-Na salt*) is intended for all animal species and categories with a minimum purity of 40% of *DLM*;
- *DL-methionine protected with copolymer vinylpyridine/styrene* (*DLM-RP-copolymer*) is intended for dairy cows with a minimum purity of 74% of *DLM*;
- *DL-methionine protected with ethylcellulose* (*DLM-RP-ethyl cellulose*) is intended for ruminants with a minimum purity of 85% of *DLM*;
- *hydroxy analogue of methionine* (*HMTBa*) is intended for all animal species and categories with a minimum purity of 88%;
- *calcium salt of hydroxy analogue of methionine* (*HMTBa-Ca*) is intended for all animal species and categories with a minimum purity of 84% of *HMTBa*; and
- *isopropyl ester of the hydroxylated analogue of methionine* (*HMBi*) is intended for dairy cows with a minimum purity of 95%.

The *feed additives* are intended to be mixed either in *premixtures* or to be added directly to complete *feedingstuffs* or *water*. The Applicant proposed no minimum or maximum *methionine* concentrations in *feedingstuffs*.

For the determination of *methionine* in *feed additives* containing *DLM, DLM-Na salt, DLM-RP-ethyl cellulose* or *DLM-RP-copolymer* the EURL identified the ring-trial validated ISO/CD 17180 method. The method applies an amino acid analyzer or reversed phase ion exchange high performance liquid chromatography equipment coupled with post-column derivatisation and spectrophotometric ultraviolet or fluorescence detection (RP-HPLC-UV/FD) for the determination of *methionine* content in commercial amino acid products. The following performance characteristics are reported for a *methionine* content ranging from 31 to 93 %:

- a relative standard deviation for *repeatability* (RSD_r) ranging from 0.5 to 1.1 %; and
- a relative standard deviation for *reproducibility* (RSD_R) ranging from 1.5 to 2.6 %.

Based on the performance characteristics presented, the EURL recommends for official control the ISO/CD 17180 method, based on ion exchange chromatography coupled with post-column derivatisation and UV or fluorescence detection to determine *methionine* in *feed additives* containing *DLM, DLM-Na salt, DLM-RP-copolymer* or *DLM-RP-ethyl cellulose*.

For the determination of *methionine* in *premixtures* and *feedingstuffs* containing *DLM, DLM-Na salt, DLM-RP-ethyl cellulose* or *DLM-RP-copolymer*, the Applicant submitted the ring-trial validated Community method - Commission Regulation (EC) No 152/2009. The following performance characteristics were reported for the determination of *total methionine*:

- RSD_r ranging from 1.1 to 5.6 %;
- RSD_R ranging from 6.9 to 13%; and
- a limit of quantification (LOQ) of 0.25 g/kg.

Furthermore, upon request of the EURL, the Applicant submitted experimental data obtained applying the above mentioned Community method for the determination of the *methionine* in *water* containing *DLM* and *DLM-Na salt*. The EURL calculated the following performance characteristics from the experimental data provided by the Applicant for *methionine* concentrations ranging from 0.5 to 10 g/L:

- RSD_r ranging from 0.1 to 2.6%;

- a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 0.3 to 2.6 %; and
- a *recovery rate* (R_{Rec}) ranging from 98.7 to 101.3%.

Based on the performance characteristics presented, the EURL recommends for official control the ring-trial validated Community method, based on ion exchange chromatography coupled with post-column derivatisation and UV or fluorescence detection to determine *methionine* in *premixtures*, *feedingstuffs* and *water* containing *DLM*, *DLM-Na salt*, *DLM-RP-ethyl cellulose* or *DLM-RP-copolymer*.

For the determination of *HMTBa* in *feed additives* containing *HMTBa* or *HMTBa-Ca*, the Applicant submitted a single laboratory validated and further verified titrimetric method. The following correspondent performance characteristics were reported:

- RSD_r ranging from 0.1 to 0.8 %;
- RSD_{ip} ranging from 0.2 to 1.1 %; and
- R_{Rec} ranging from 99.7 to 101.1 %.

Based on the performance characteristics presented, the EURL recommends for official control the single laboratory validated and further verified titrimetric method to determine *HMTBa* in *feed additives* containing *HMTBa* or *HMTBa-Ca*.

For the determination of *HMTBa* in *premixtures* and *feedingstuffs* containing *HMTBa* or *HMTBa-Ca*, the Applicant submitted a single laboratory validated and further verified method based on RP-HPLC-UV. The following correspondent performance characteristics were reported for concentrations in *premixtures* and *feedingstuffs* ranging from 5 to 80 g/kg and from 0.14 to 4 g/kg, respectively:

- RSD_r ranging from 0.7 to 2.2 %;
- RSD_{ip} ranging from 1.7 to 8.3 %;
- R_{Rec} ranging from 96 to 108 %; and
- LOQ of 0.2 and 0.08 g/kg *feedingstuffs* for *HMTBa-Ca* and *HMTBa*, respectively.

Based on the performance characteristics presented, the EURL recommends for official control the single laboratory validated and further verified RP-HPLC-UV method to determine *HMTBa* in *premixtures* and *feedingstuffs* containing *HMTBa* or *HMTBa-Ca*.

The Applicant provided no experimental data for the identification of *HMTBa* in *water*. Therefore the EURL cannot evaluate nor recommend a method for official control to determine *HMTBa* content in *water*.

For the determination of *HMBi* in the *feed additive* and *feedingstuffs* the Applicant submitted a single laboratory validated and further verified analytical method based on RP-HPLC-UV. The following performance characteristics were reported:

- RSD_r ranging from 0.2 to 3.7 %;
- RSD_{ip} ranging from 0.4 to 4.6 %;
- R_{Rec} ranging from 94 to 104.7 %; and
- LOQ of 2.3 g/kg in *feedingstuffs*.

Based on the performance characteristics presented, the EURL recommends for official control the single laboratory validated and further verified RP-HPLC-UV method to determine *HMBi* in the *feed additive* and in *feedingstuffs*.
Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by Article 10 (Commission Regulation (EC) No 378/2005) is not considered necessary.

APPENDIX B

Absorption, distribution, metabolism and excretion of methionine and its analogue

After breakdown of dietary protein by peptidases, the released L-methionine (L-Met) is absorbed by sodium-dependent (e.g. neutral brush border (NBB) or Phe transporter) and sodium-independent transport systems in the brush border membrane. In pigs fed a corn soybean meal-based diet, the apparent ileal and standardised ileal digestibility of methionine is about 86 % and 90 %, respectively (e.g. Stein et al. 2007). The apparent ileal digestibility of methionine in feed ingredients varies, ranging from about 65 % for lupin seeds, through 72–86 % for grains and grain products and 86–88 % for soybean products, sunflower meal and corn gluten meal, to about 92 % in milk and whey. Digestibility and bioavailability of crystalline L-Met, ignoring potential microbial degradation in the intestine, is thought to be about 100 %, as for all crystalline amino acids (e.g. Izquierdo et al. 1988; Han et al., 1990; Chung and Baker, 1992). The intestinal uptake of the DL-hydroxy analogue of methionine (HMTBa) is mediated by sodium-independent and by H^+- and partially Na^+-dependent transport systems and may also occur by diffusion along the small intestine (Brachet and Puigserver 1987, 1989; Maenz and Engele-Schaan 1996; Richards et al., 2005; Martín-Venegas et al. 2006a, 2007, 2008). Commercial HMTBa contains significant amounts of oligomeric HMTBa forms, which are evidently effectively hydrolysed in the small intestine, because HMTBa absorption from sources containing predominantly monomers or oligomers appears to be similar (Martin-Venegas et al., 2006a). Studies in chicks indicate that the absorption rates of L-Met and HMTBa are quite similar, particularly at low concentrations (Knight and Dibner, 1984). The intestinal absorption of DL-HMTBa was 95.9 % in caecectomised and 98.8 % in sham-operated conventional cockerels compared with 99.7 % for DL-Met in both groups, with a DL-HMTBa bioavailability relative to DL-Met of 91.3 % ± 11.8 % (Han et al., 1990). In a study of tritiated L-Met and L-HMTBa absorption in chickens (Drew et al., 2003), it could be shown the proportion of labelled material in the distal ileum originating from L-methionine (< 4 %) was lower than that originating from L-HMTBa (about 10 %). Comparable results were obtained in pigs (Malik et al., 2009). Both studies indicate that microbial degradation of L-HMTBa in the small intestine is higher than that of L-Met.

The hydroxy analogues of amino acids are converted to the L-isomer of the amino acid in two steps. D-Met and the D- and L-hydroxy analogues of methionine are first stereospecifically oxidised by a D-2-hydroxy acid dehydrogenase and L-2-hydroxy acid oxidase, respectively, to form the keto analogue of methionine, 2-keto-(4-methylthio) butanoic acid (KMB); this keto intermediate is then transaminated to L-Met by different donor amino acids (e.g. Gordon and Sizer, 1965; Dibner and Knight, 1984; Dupuis et al., 1989, 1990; Ferjancic-Biagini et al., 1995; Rangel-Lugo and Austic, 1998; Martín-Venegas et al., 2006b). HMTBa-converting enzymes can be found in various tissues, e.g. intestine, liver, kidney.

Methionine is mainly used for protein synthesis or is alternatively metabolised to homocysteine via *S*-adenosyl-methionine and *S*-adenosyl-homocysteine or transaminated to 3-methylthiopropionate and then to volatile sulphur compounds (methanethiol and/or hydrogen sulphide). Homocysteine can further be converted to cystathione and then to cysteine or it may be recycled to methionine using 5-methyltetrahydrofolate or betaine (from diet or choline oxidation) as the methyl donor. The metabolic pathways of methionine and related compounds are outlined in Figures 6 and 7.

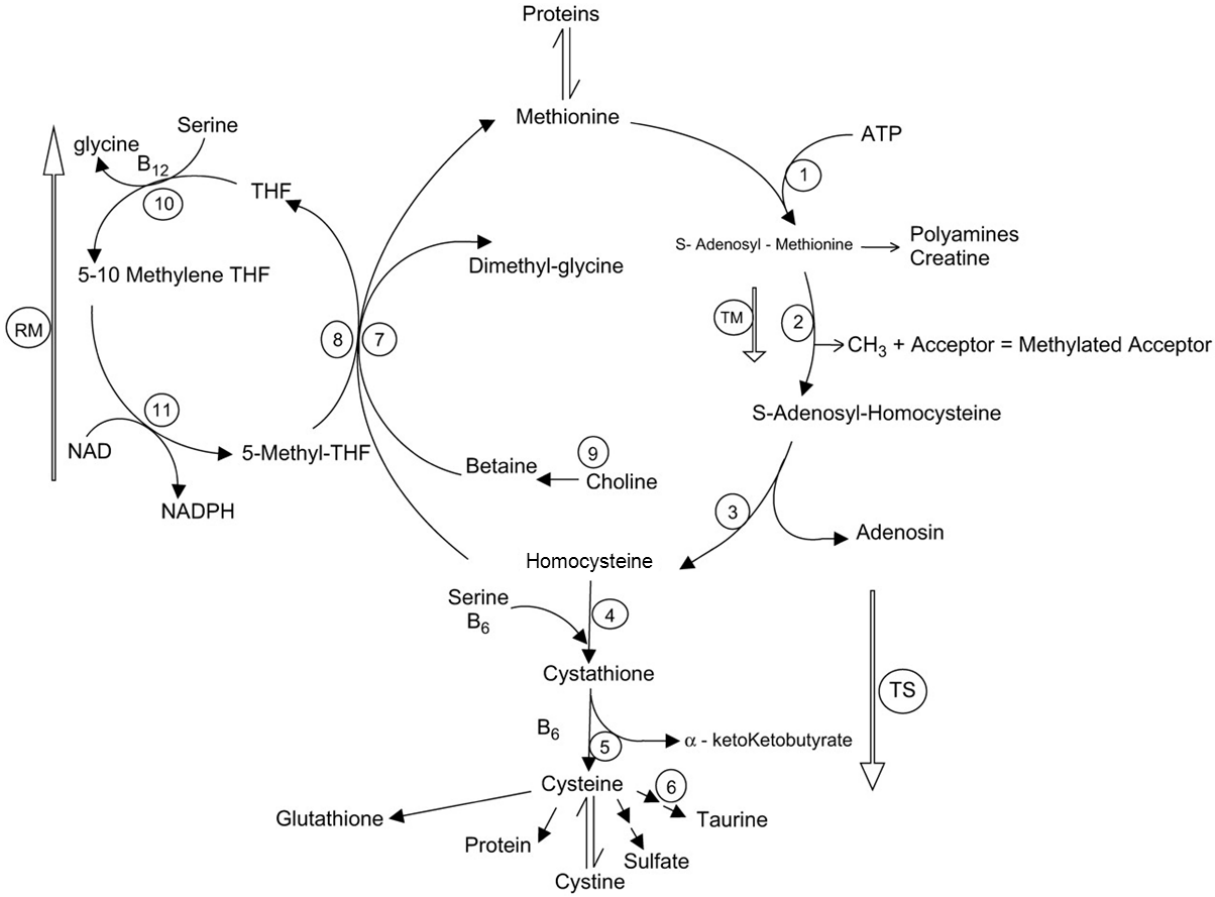

Figure 6. The major metabolic pathways of methionine (Ball et al., 2006)
TM, transmethylation, TS, transsulphuration, RM, remethylation.

Transmethylation and transsulphuration are the major pathways for the catabolism of methionine, whereas the transamination processes appear to become more significant at excessive methionine intakes leading to methionine toxicity (e.g. Cooper 1983, 1989; Brosnan and Brosnan 2006).

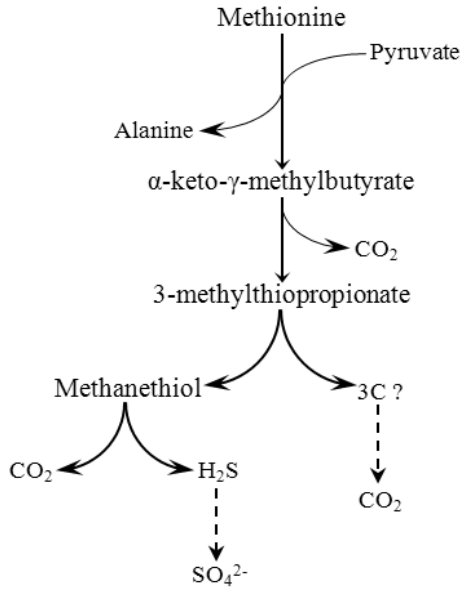

Figure 7. The transamination pathway of the methionine metabolism
(According to Finkelstein and Benevenga, 1986)

ABBREVIATIONS

DL-Met: DL-methionine.

DL-Met-Na: DL-methionine sodium salt.

HMTBa: hydroxy analogue of methionine.

HMTBa-Ca: calcium salt of hydroxy-analogue of methionine.

HMTB-i: isopropyl-ester of methionine hydroxy analogue.

DL-Met-cop: DL-methionine technically pure protected with copolymer vinylpyridine/styrene.

DL-Met-ec: DL-methionine technically pure protected with ethylcellulose.