

Organic forms of trace elements as feed additives: Assessment of risks and benefits for farm animals and consumers*

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Abstract: Essential trace elements (TEs) are major nutritional feed additives, required for the health and welfare of farm animals, especially under intensive husbandry conditions. Within the European Food Safety Authority (EFSA), risk assessment of feed additives is carried out by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), which is increasingly confronted with additives based on organic forms of TEs. Organic forms are expected to have higher bioavailability, hence, lower levels in feeds may be needed to meet farm animal requirements. On the other hand, higher bioavailability might also lead to enhanced deposition in edible tissues, hence, increased consumer exposure; specific organic forms might also have a different distribution in edible tissues or products. Higher consumer exposure is especially relevant to TEs with recognized health risks at excess levels (e.g., Se); exposure scenarios should take into account background dietary levels and other intake sources (e.g., supplements). Tolerable upper intake levels (ULs) for adults and children are the basis for risk assessment; when no UL is defined (e.g., for Mn), a conservative estimate should assess whether the organic form in feeds might significantly increase the consumer's intake above the dietary background. In perspective, specific maximum allowable contents might be considered for organic forms of TEs in feeds, when supported by a robust database as well as by targeted analytical methods.

Keywords: bioavailability; chromium; exposure; farm animals; food safety; manganese toxicology; selenium.

INTRODUCTION

Feed additives are a large and heterogeneous group of substances, from enzymes through to nutrients, used to improve the quality of feeds for farm animals: they make up the bulk of chemicals used in farm animal production and may significantly influence the wholesomeness of foods of animal origin [1]. Since 2003, the risk assessment of feed additives in Europe has been carried out by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) within the European Food Safety Authority (EFSA) [2]. The assessment of feed additives is a complex, multidisciplinary task [1]. The substance should be credible from the efficacy standpoint; tolerance studies in target species should assess the margin of safety between the intended concentrations in feed and the level where initial un-

*Paper based on a presentation at the 3rd International Symposium on Trace Elements in Food (TEF-3), 1–3 April 2009, Rome, Italy. Other presentations are published in this issue, pp. 349–481.

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desirable effects are seen. It is most important to assess the possible carry-over to consumers through edible tissues and products, and derive a conservative estimate of the consumer's intake; this has to be weighted against the toxicological characterization of the substance. The assessment is not confined to target species and consumers, as external impacts of the food chain need to be evaluated, such as possible safety concerns for users and for the environment. The assessment may have specific features for different additive groups, e.g., nutrients, such as vitamins or trace elements (TEs), used in nutritional additives. Whereas xenobiotics, such as coccidiostats, require full toxicological testing and the definition of acceptable daily intake, nutritional additives are assessed on the basis of their tolerable upper intake levels (ULs): one example is the comprehensive FEEDAP opinion on iodine salts [3]. A UL is the highest level of daily nutrient intake that is likely to pose no risk of adverse health effects for almost all individuals in the general population [4]; in the European Union (EU) risk assessment framework, most ULs for TEs were defined before the EFSA establishment by the Scientific Committee on Food (SCF) [5]. Moreover, contrary to xenobiotics, for nutritional additives it is purposeless to establish maximum residue limits in edible tissues and products, since it would be extremely difficult to distinguish, under practical conditions, the contribution from feed additives and the natural background [6]. As a consequence, the control of correct and safe use of nutritional additives relies mostly on feed monitoring plans and good farming practices. Subclinical deficiencies of TEs are important in farm animals: the main causes are environmental deficiencies related to soil quality and/or intensive husbandry conditions. For instance, breeds selected because of high-quality or high-output production may have enhanced requirements, because of the lower feed conversion rate; also, less than optimal intensive farming may increase nutrients requirement to support adequate immune function. Indeed, inadequate intakes of essential TEs seldom lead to clinical deficiency syndromes; instead, they may significantly impair production, reproductive capacity, or the ability to cope with stress and infections [4,6,7]. Accordingly, TEs feature prominently amongst feed additives with nutritional purposes.

ORGANIC FORMS OF TRACE ELEMENTS IN FEEDS: PRINCIPLES AND CRITERIA FOR ASSESSMENT

The use of TEs to integrate the nutritional aspects of feeds is well established in farm animal husbandry; many TE salts (Cu, I, Mn, Se, Zn, etc.) have been long authorized for use in Europe. TE-based additives must be supplemented in compliance with the maximum authorized levels of the total element (i.e., background plus additive) in feeds. Such maximum authorized levels have been established in the last decades according to available data on nutritional requirements and tolerance in farm animals, as well as allowances accounting for, e.g., losses due to technological processing [4]. Maximum levels are also expected to guarantee toward unwanted carry-over to consumers; however, FEEDAP recommended in 2005 the reduction of maximum authorized levels in feeds of iodine based on a new assessment of potential consumer exposure [4]. Other TEs not recognized as essential have been proposed in farm animal nutrition for zootechnical purposes, in order to support and improve farm animal production. Major examples are organic compounds of Cr(III), used to ease stress and improve meat production in pigs [8], and organic compounds of As (*p*-arsanilic acid and 4-hydroxy-3-nitrophenyl-arsonic acid-roxarsone) used as feed additives in poultry and swine intensive farming for controlling protozoan infections and enhancing feed efficiency [9]; neither Cr(III)- nor As-based additives are authorized in Europe.

Since 2006, however, FEEDAP has seen a changing scenario with an increasing number of dossiers on organic forms of TEs. Currently, FEEDAP has finalized eight comprehensive assessments during the 2006–2009 period, including three opinions on selenized yeasts [6,10,11], two opinions on a Zn chelate of hydroxy analog of methionine, namely, one general opinion and a specific opinion on its safety for fattening chickens [12,13], and one opinion each on Cu and Mn chelates of hydroxy analog of methionine [14,15] and Cr(III) methionine [8], all intended as nutritional additives (see Table 1 for a synopsis).

Table 1 Synopsis of the opinions on organic forms of TEs intended as feed additives published by EFSA until June 2009.

Trace element	Organic form	Maximum allowed concentrations in feed of the total TE (EU)	Proposed target animal species for the additive	References
Cr(III)	Cr(III)methionine complex	<i>No maximum allowed concentration for Cr(III) in EU:</i> proposed concentration between 0.4 (pigs) and 1.6 (calves, lambs, kids) mg added Cr(III)/kg complete feedingstuff	All species	[8]
Cu	Chelate with hydroxy analog of methionine	25 mg/kg complete feed, except for: calves and ovine, 15 mg/kg; other cattle, 35 mg/kg; crustaceans, 50 mg/kg; piglets, 170 mg/kg	All species	[14]
Mn	Chelate with hydroxy analog of methionine	100 mg/kg for fish and 150 mg/kg for other species	All species	[15]
Se	Selenized yeast (>60 % selenomethionine)	0.5 mg/kg complete feed	Cattle, pig, poultry	[6]
	Selenized yeast (>60 % selenomethionine)	0.5 mg/kg complete feed	All species	[10]
	Selenized yeast (>60 % selenomethionine)	0.5 mg/kg complete feed	All species	[11]
Zn	Chelate with hydroxy analog of methionine	150 mg/kg complete feed, except: 200 mg/kg milk replacers and fish feed; 250 mg/kg pet feed	All Chickens for fattening	[12,13]
				[13]

Organic forms are expected to be more bioavailable in comparison with inorganic salts. Indeed, while the occurrence of compound-specific beneficial effects might be considered in some cases, bioavailability of intended nutrients is the one key parameter to assess the efficacy of nutritional additives [16]. When proven, increased bioavailability may have a substantial advantage, as lower levels in feeds could be needed to meet nutritional requirements; in its turn, lower levels in feeds would also lead to lower environmental output (from animal excreta). Indeed, a reduction of environmental output may be desirable for, e.g., Cu, which has high requirement levels in pigs but is also highly toxic to small ruminants grazing on pastures contaminated by pig excreta [17]. On the other hand, higher bioavailability, i.e., higher systemic access in bioactive form, could also lead to increased carry-over to consumers through enhanced deposition in edible tissues and products, such as eggs and milk [18]. Therefore, the assessment of organic TEs has to confront a series of specific issues.

To prove the credibility of the effectiveness claim, the additive should deliver the essential TEs in a bioaccessible way, as shown in at least one adequate study on a relevant farm animal species, or even

in a laboratory species [6]; the key studies on effectiveness should include, as a comparative “standard”, an inorganic form authorized as feed additives, e.g., Na selenite or Cu sulfate [6,10,11,14]. Thus, for nutritional additives bioavailability of the intended essential nutrient is the only parameter that does matter. As mentioned above, bioavailability is not limited to increased absorption and/or tissue deposition; instead it has to be measured through the appropriate biomarkers for that specific element, such as liver concentration of Cu or bone and liver concentrations of Zn in monogastrics and ruminants, respectively [12,14]. Table 2 presents a synopsis of bioavailability parameters, as identified by FEEDAP for the organic forms of TEs assessed until now. Moreover, bioavailability has to be assayed at concentrations compatible with the maximum total (i.e., additive plus background) content in feed allowed in the EU (Table 1). It might also be noteworthy that such maximum contents currently do apply to the TE itself, irrespective of the chemical form; moreover, they may change in total feeds for different species, e.g., for total Mn the maximum allowed content is 100 mg/kg for fish and 150 mg/kg for all the other species allowed [15].

Table 2 Parameters applied to assess bioavailability of organic forms of TEs in farm animals.

Trace element	Biomarker(s)	Farm animal species	References
Cr(III)	Increased blood glucose clearance rate	Pigs, beef cattle	[8]
	Reduced plasma glucose	Horses and dairy cows	
	Reduced insulin	Horses	
Cu	Liver Cu concentrations	All	[14]
	Cu chaperone for Cu/Zn superoxide dismutase (biomarker of Cu deficiency)		
Mn	Bone Mn concentrations	All	[15]
Se	Se concentrations in liver, whole blood or in plasma/serum; glutathione peroxidase activity in whole blood, erythrocytes or in plasma	All	[10]
Zn	Bone and pancreatic Zn concentrations	Monogastrics	[12]
	Liver and kidney Zn concentrations	Ruminants	

Toxicological studies include tolerance studies in farm animals as well as a limited toxicological dataset. The aim of tolerance studies is to assess whether the organic form may change (e.g., through enhanced bioavailability) the margin of safety established for the TE; again, comparison with an authorized, bioavailable inorganic form should support the assessment. Conclusions on one species should be extended to other species with caution: for instance, the Mn, Cu, and Zn chelates of hydroxy analog of methionine are shown to be well tolerated in broiler chickens, which are quite resistant to the toxicity of these elements. Therefore, tolerance findings on chickens could not be extended to other, more sensitive, species for which studies were not presented, e.g., sheep which are highly sensitive to Cu toxicity [12–15]. A limited, basic toxicology dataset (in vitro/in vivo genotoxicity and 90-day rodent study)

is also requested. Toxicological effects are not expected to change qualitatively as compared to inorganic forms; thus, the toxicological dataset in farm animals, laboratory animals, and in vitro systems primarily intends to show whether there are any unexpected increases of toxicity. Noticeably, up to now the organic forms assessed by FEEDAP did not show major changes as regards tolerance in target species or in vitro/in vivo toxicology [6,8,10–15]. Sometimes, effects of concern may be identified, e.g., Cu hydroxy analog of methionine significantly increased chromosome aberrations in vitro: upon comparisons with literature data, the genotoxic effect was similar to those produced by other Cu sources, thus, FEEDAP considered that no additional or specific toxicity concern was introduced by the Cu chelate [14]. One noticeable exception are the organic compounds of As, such as roxarsone, not authorized in the EU: these produce specific neurotoxic effects in pigs and poultry, with roxarsone characteristically targeting spinal cord, whose onset is modulated by the intake of Cu and cysteine [19]. A recent in vitro study showed that roxarsone has an angiogenic effect of comparable potency as inorganic arsenite [As(III)], but it acts through different mechanisms [20]. Organoarsenicals appear as a peculiar instance, rather unmatched among essential elements; however, it cannot be ruled out altogether that other organic forms might have unique effects.

Consumer carry-over includes how much the organic form is adding up or altering the pattern of the carry-over due to background content in feed and to the already authorized inorganic forms. Consumer exposure can be assessed only for the use on those major species for which adequate studies do exist; for instance, FEEDAP opinions until now could not assess adequate studies on carry-over in farmed fish, which may have metabolic rates slower than as well as dependent on the growth stage [21]. Most important, tissue deposition studies should include, when relevant, dairy cows and laying hens: these have longer life than meat-producing animals, thus allowing prolonged bioaccumulation, and there may be specific excretion patterns of individual TEs relevant to milk and/or eggs [22,23].

The first tier of consumer exposure assessment relies on a highly conservative, worst-case scenario with theoretical, default daily consumption figures, corresponding to: 300 g of meat (or fish flesh), 100 g of liver, 50 g of kidney, 100 g of egg, and 1500 g milk [6]. To this worst-case intake, the background intake from diet and possibly other sources have to be added [6,8,10–12,14,15]. The scenario has to be weighted against the established UL in Europe for the element (Table 3). If there is a chance identified to exceed the UL based on the worst-case scenario, a more refined consumer exposure assessment may be performed [6,10,11].

Table 3 ULs for adults and children (4–6 years) for TEs whose organic forms have been assessed by EFSA FEEDAP.

Trace element	Adult UL	Children (4–6 years) UL	References
Cr(III)	None	None	[8]
Cu	5 mg/day	2 mg/day	[14]
Mn	None	None	[15]
Se	0.3 mg/day	0.09 mg/day	[6]
Zn	25 mg/day	10 mg/day	[12]

Environmental risk assessment of organic forms of TEs has not been identified as a major problem until now. TEs are naturally present in the environment, but concerns might arise when their concentration or distribution are altered. However, organic forms will replace other sources of TEs; moreover, when bioavailability is actually enhanced, lower levels of organic forms would actually be required in farm animal nutrition practice. Therefore, provided that total maximum authorized levels of the specific TE in feed are not exceeded, the use of an organic form will not present an additional load nor an additional risk to the environment compared to other sources of the TE for which it will substitute [6,10,11].

EXAMPLES OF RISK ASSESSMENT OF ORGANIC FORMS OF TRACE ELEMENTS AS FEED ADDITIVES

Selenized yeasts

Three out of seven organic forms of TEs whose assessments have been finalized by FEEDAP are selenized yeasts, possibly because of the remarkable marketing interest toward bioavailable and safe Se supplements in animal farming [6,10,11]. Inorganic Se compounds are already authorized as feed additives in Europe in the forms of sodium selenite and sodium selenate, but organic forms have not been authorized until the EFSA assessments. In any case, the use of whatever Se-based supplement must comply with the maximum authorized level of 0.5 mg total Se in complete feeds (with a moisture content of 12 %). The three additives presented essentially the same features and issues, thus, for the sake of brevity, they are considered together here. The products are composed by inactivated and dried cells of different strains of the yeast *Saccharomyces cerevisiae*: in the whole product, Se is present at concentrations ranging 0.20–0.24 %. Selenomethionine represents approximately 63 %, and in all cases less than 70 % of the total Se; inorganic Se represents at the most 3 % (but most often ≤ 1 %), almost exclusively Se(IV). The remaining Se, approximately 30 %, was composed in all three cases by unidentified organic Se compounds; selenocysteine was also determined in one case, but it accounted for 2–4 % only [10]. Accordingly, FEEDAP recommended a better characterization of this significant fraction in all three cases [6,10,11]. One selenized yeast [6] was intended as Se source for some major species (poultry, pigs, and cattle); the remaining two additives [10,11] were intended for all farm animals, but in fact the database on tolerance and tissue deposition was adequate only for use in poultry, pigs, and cattle. Selenium status was assessed using consolidated biomarkers: Se concentrations in liver, whole blood, or plasma/serum, and glutathione peroxidase activity in whole blood, erythrocytes, or plasma/serum [10]. All three selenized yeasts were shown to be bioavailable; overall, the bioavailability was either comparable or somewhat increased compared to Na selenite, with no major changes. The safety margin was also not altered as compared to that expected from inorganic sources, as the supplementation above 10 times the authorized maximum Se content of feeds reduced production parameters of poultry, pigs, and cattle. Genotoxicity assays and toxicity studies confirmed that none of the selenized yeasts introduced any additional toxicity compared to other sources of Se. However, the tissue deposition was changed, as there was a significantly higher deposition of Se in muscle and, to a lesser extent, in egg white and milk protein, as compared to the inorganic sources. This was attributed to the unspecific incorporation of selenomethionine into proteins, where it is interchangeable with sulfur containing methionine as well as Se reservoir [6]. Noticeably, no information could be derived on the Se species involved in tissue deposition.

The SCF assessment of Se defined the average background intake of Se from foods other than farm animal products (fish and vegetables) as 60 $\mu\text{g/day}$ in European adults. The adult UL is 300 $\mu\text{g/day}$: age-adjusted ULs were derived by SCF on body weight basis, e.g., for small children (4–6 years) 90 $\mu\text{g/day}$ [6]. First-stage consumer exposure assessment was performed using the theoretical worst-case figures of daily consumption and the expected Se contents in milk, eggs, and edible tissues from pigs (the species with the highest tissue level) upon supplemental addition to the feeds up to the maximum permitted level: although to a somewhat variable extent, also depending on the strength of the respective datasets, the resulting figures were above the UL for the three selenized yeasts [6,10,11]. To refine consumer exposure, FEEDAP used more realistic consumption figures for adults derived from a EU survey, i.e., 105 g/day meat, 280 ml/day milk, and 36 g/day egg: liver and kidney figures were derived from the meat figures, using the same proportion as in the theoretical worst-case calculation, resulting in 35 and 3.5 g/day for liver and kidney, respectively. Consumption figures were estimated also for 4–6-years old children, as a potentially vulnerable consumer group, as follows: half the quantity of meat products and eggs consumed by adults, no offal, and 250 ml of milk. The refined consumer exposure assessment resulted in total (i.e., including background) Se slightly above the children UL, whereas it was always far below the adult UL: for instance, in the case of the selenized yeast

Sel-Plex the calculated total intake was 99 µg/day (110 % of UL) for children and 165 µg/day (55 % of UL) for adults [6]. However, the background dietary intake has been calculated for adult food consumption figures; FEEDAP assumed that background Se intake in small children is expected to be significantly lower, thus, the total Se intake would most likely be well below the specific UL also for this vulnerable age group. Although the exposure assessment using average consumption figures might have somewhat overlooked “extreme consumers”, sufficient conservativeness was ensured by using Se concentrations derived from the species with the highest tissue levels upon addition in feeds up to the maximum allowed Se level. Accordingly, the use of selenized yeasts would not cause any risk to consumers, provided that the maximum Se content allowed in feeds is not exceeded.

Cr(III)-methionine

The assessment of Cr-methionine as nutritional additive for all species has been completed in 2009 by FEEDAP; since Cr is not allowed as a TE in farm animal nutrition, the specific assessment of the organic chelate of TE required a preliminary, comprehensive evaluation of the possible role of Cr(III) as nutritional additive as well as of the related risks [8].

The background Cr(II) in foodstuffs comes mainly from mineral sources and only to a limited extent from plant sources. Cr(III) has a recognized endocrine-metabolic action, potentiating insulin-dependent glucose entry into the cells as well as, albeit with a lower level of evidence, modulating immune response, leptin balance, and lipid metabolism [24]. However, no symptoms of Cr(III) deficiency have been demonstrated either in animals or humans; therefore, there is no evidence of essentiality and no requirements can be established. Accordingly, Cr(III) cannot be considered as a nutritional additive, although there is a wealth of studies intended to demonstrate favorable effects in farm animals: such favorable effects are especially evident in animals under stress conditions and depend also on natural Cr(III) background in feed, the sources and levels of supplementary Cr and the presence of other dietary factors, including other TEs such as Fe, Mn, V, and Zn [8]. Analogous limitations and uncertainties do not allow assessment of the maximum tolerable levels of supplemental Cr(III) in feed based on tolerance in farm animals. It is also difficult to assess Cr(III) bioavailability due to the lack of established biomarkers. The toxicology of Cr(III) is not yet fully clarified. Cr(III) is much less toxic than Cr(VI), an environmental contaminant and an established carcinogen, but recent literature suggests a possible genotoxic potential *in vivo* [25,26]; undesirable endocrine-metabolic effects may also be considered [24]. It is noteworthy that the SCF was unable to establish a UL when assessing Cr(III) in 2003, due to information inadequacies [27]. Taking into account concerns and uncertainties, FEEDAP considered it prudent to avoid any additional exposure of the consumers resulting from the use of supplementary Cr in animal nutrition. Consumer carry-over from Cr(III) in feeds is also difficult to assess since the available data do not show a consistent pattern of tissue deposition, also due to significant analytical uncertainties [8]. The consumer background dietary intake of Cr(III) is likely to be around 0.1 mg/day and not expected to exceed 0.3 mg/day: the contribution of foodstuffs of animal origin from unsupplemented Cr(III) animals to the background dietary intake for adults is estimated in the range of 16–26 %, mainly attributable to offals, followed by muscle (including fish) and eggs [8,27,28]. However, no reliable data are available to assess the additional consumer’s exposure resulting from the use of supplementary Cr in feeds. Finally, FEEDAP notes that concerns about Cr(III) toxicity, and especially genotoxicity, should alert also about user’s safety, so that occupational exposure in the feed industry should be kept to a minimum.

Against this, rather unfavorable, background, FEEDAP carried out the specific assessment of the organic TE compound, Cr(III)-methionine; actually, the compound contains 3 % Cr(III) and is a chloride salt of a hexacoordinate chelate complex of a Cr(III) atom with three methionine molecules. The intended supplementation level was 0.4–1.6 mg/kg complete feedstuffs as Cr(III). Overall, dietary Cr is poorly absorbed by animals (≤ 1 % of the ingested dose): limited evidence suggest that organic forms of Cr(III) are better absorbed, such as Cr-nicotinate, Cr-picolinate, or Cr-yeasts, in the range 1.5–10 %

of the ingested dose. Cr(III)methionine was considered a bioavailable source of Cr(III), based on several biomarkers related to glucose metabolism, as the main biological target of Cr(III), namely: increased glucose clearance rate in pigs and beef cattle, reduced plasma glucose in horses and dairy cows and reduced insulin in horses. A tolerance study in pigs did not show any significant effects, whereas one feeding study on cows suggested a negative effect on milk production at near-use levels; thus, the limited data did not allow the conclusion that the enhanced bioavailability of Cr(III)-methionine was of no consequence for farm animal safety.

Genotoxicity was a point for clarification, due to the relationship between effect and bioavailability, Cr(III) is the likely ultimate intracellular form of carcinogenic Cr(VI); Cr(III) itself has very low intracellular accessibility, but this might be significantly enhanced for organic forms [29]. Indeed, a recent study showed a markedly higher reactivity with cell proteins of organic form Cr(III)picolinate as compared to inorganic forms. The question has not been solved by the studies provided on Cr(III)-methionine; they did yield negative results, but were considered inconclusive since the end-points typical of Cr(VI) genotoxicity (e.g., oxidative DNA damage, DNA adducts) were not specifically assessed. Tests showing Cr(VI) genotoxic effects in direct comparison with Cr(III)-methionine should have been provided, instead. Finally, the critical issue of consumer exposure assessment could not be solved because of the inadequacies of the studies performed. It was possible only to assess that no significant carry-over occurred in bovine milk; however, milk itself is not a significant dietary source of Cr(III), as compared to offals, meat, or eggs [8,27]. Overall, the data were inadequate to deal with the main questions associated with enhanced bioavailability of Cr(III), such as genotoxicity and tissue deposition; thus, no conclusion could be made on whether the use of Cr(III)-methionine in farm animal feeds would result in any different concern for consumer safety compared to other Cr(III) sources. As discussed above, Cr(III), and Cr(III) methionine, cannot be considered as nutritional feed additives, due to the lack of demonstrated essentiality; however, the need for proper data on consumer exposure and safety remain unchanged also considering other potential uses, e.g., production enhancers, in farm animal nutrition.

It is noteworthy that FEEDAP has adopted toward *an organic form of Mn* (Mn chelate of hydroxy analog of methionine) an approach basically similar to that adopted in Cr(III)-methionine [14] assessment. Manganese is a recognized essential nutrient in farm animals; however, no UL has been established by the SCF [30]. Differently from Cr(III), the UL was not established due to insufficient data as well as remaining concerns about oral neuro- and genotoxicity: accordingly, the SCF advised that additional dietary exposure to Mn above the dietary background may carry a health risk [30]. As in the case of Cr(III), the approach adopted by FEEDAP was that the use of the organic Mn supplement in feeds should not lead to any significant increase of Mn intake by consumers. Inorganic sources of Mn are already authorized in animal nutrition: the limited dataset presented on the Mn chelate did not suggest an increased bioavailability (assessed from bone Mn concentration) as compared to inorganic Mn compounds such as Mn oxide and sulfate. However, the evidence was totally inadequate to assess the carry-over to the full range of edible tissues/products, since the organic Mn form was proposed for use in all farm animal species. Thus, FEEDAP could not conclude on the safety for consumers of Mn chelate of hydroxy analog of methionine.

The opinions of the EFSA panels can include also recommendations. Noticeably, the opinions of FEEDAP on organic forms of TEs do share the recommendation about the development of analytical methods capable of distinguishing between organic and inorganic sources of the respective TEs. FEEDAP further specifies that such a recommendation would support the setting of maximum levels in feeds which can account for different bioavailability [6,8,10–15].

DETERMINATION OF ORGANIC FORMS OF TRACE ELEMENTS AS FEED ADDITIVES: PRINCIPLES AND PROBLEMS

The growing use of organic forms of TEs in animal nutrition highlights the need for selective analytical methods for these compounds. Today, when an organic source of a TE (e.g., a metal chelate with a biomolecule) is proposed as a nutritional or zootechnical additive, the manufacturer's method of analysis only concerns the total concentration of the TE. Such methods are inadequate for several reasons. First, they do not allow one to check the quality of the production process in terms of identity of the active compound (the metal chelate, in our example). Even though the total concentration complies with the manufacturer's specification, the element of interest may be partially (or even entirely) present as a less bioavailable inorganic form, and there is no means to assess this without an analytical method that selectively detects the relevant organic species. Other organic species of the element, different from those declared in the label and with different biological properties, could be present as impurities, and again this can only be ascertained through a species-selective analytical method. Apart from quality control of the production process, the exact characterization of mineral supplements is the prerequisite to the understanding of the absorption mechanism(s). Therefore, much more emphasis than hitherto has to be placed on the accurate characterization of mineral additives.

When the additive is incorporated in the final feed, analytical methods that discriminate the exogenous (added) metal or metalloid species from the TE background, i.e., the variety of endogenous naturally occurring metal(loid) species present in the feed matrix, are needed as well. The characterization of the entire set of element species (i.e., exogenous and endogenous) can greatly aid the evaluation of the studies on bioavailability and tissue deposition; these can be difficult to interpret since the variety of metal (metalloid) species and their relative proportions are seldom known.

Analytical methods for the determination of TE species have been widely applied to food and food additives [31–33]. The analytical approach is usually based on coupled (hyphenated) techniques, i.e., on the on-line combination of a chromatographic separation with an element-specific detection technique. High-performance liquid chromatography (HPLC) is commonly used as separation technique, capillary electrophoresis (CE) and gas chromatography (GC) being favored for specific applications. Inductively coupled plasma-mass spectrometry (ICP-MS) is the element-specific detector of choice in hyphenated methods owing to its easy coupling with chromatography and its high detection power, multi-element capability, wide linear dynamic range, high sample throughput, and ability to discriminate between isotopes. Molecular mass spectrometry (typically, electrospray mass spectrometry, ES-MS) is employed for confirmation of species identification carried out by retention time matching with analytical standards in HPLC-ICP-MS and for the identification of unknown species for which analytical standards are not available. Direct determination of element species by HPLC-ESI-MS(MS) is generally not feasible owing to the lower detection power of this technique compared to HPLC-ICP-MS.

The application of analytical methods based on hyphenated techniques for element speciation to feed additives has not been widespread. Most of the literature is devoted to As speciation and addressed both naturally occurring [34–36] and synthetic arsenocompounds such as the feed additives *p*-arsanilic acid and 4-hydroxy-3-nitrophenyl-arsonic acid (roxarsone) [37–40]. As far as essential TEs are concerned, most of the work on chemical speciation dealt with Se. The metalloid Se, like As, exists in nature in the form of a variety of organic compounds, which contain a covalent bond between the metalloid atom and a C atom. These are selenoamino acids, selenopeptides, and selenoproteins, which, along with the inorganic species (selenite and selenate), constitute the set of naturally occurring Se species that can be found in feed. Selenized yeast, which is produced by fermenting yeast in an Se-enriched medium, thus causing the Se to become organically bound to yeast components (mainly proteins), is the vehicle currently used for supplementing the diet of farm animals with organoselenium compounds [6,10,11]. Se speciation in selenized yeast has been extensively studied, especially in the last decade; selenomethionine is the most abundant Se species in enzymatic extracts of selenized yeast, accounting

for $\geq 60\%$ of the total Se content [41]. HPLC-ICP-MS has been the analytical approach commonly used in these studies following proteolytic extraction for liberation of selenoamino acids from proteins; separation of Se species was achieved mainly through ion-pairing reversed phase, cation-exchange and anion-exchange chromatography [31–35]. Most recent studies employed molecular mass spectrometry for structural confirmation/identification. However, products described as Se-yeast may show a remarkable variability concerning their species composition [41]. In good-quality products, the percentage of organically bound Se should be greater than 90 %, and more than 80 % should be bound to yeast proteins, including cell-wall proteins. However, in some products, the percentage of sodium selenite is such that most of the Se is clearly not bound to the yeast; at worst, there may merely be a mixture of sodium selenite and yeast, the Se not being bound to yeast biomolecules [41]. It may be mentioned here *passim* that the issue of undesirable impurities in mineral-based feed additives and ingredients may be a serious one: examples include the Pb contamination of the Mn inorganic source manganomanganic oxide [42] and the significance presence of dioxins in mineral feed additives such as sepiolite, even though the latter seem poorly bioavailable [43].

Differently from Se, the organic forms of the other essential TEs (Cu, Fe, Mn, Zn) used as feed additives are coordination complexes in which the metal atom is coordinated by one or more bioligands. The bioligands are typically 2 or 3, usually amino acids such as glycine or methionine, and a chelate complex is formed [44,45].

Very little analytical work has been done in the area of metal complexes in feed. Cr speciation has been addressed in an early work [46], but the focus was on the determination of soluble Cr(VI) compounds in feed, irrespective of the bioligands involved, due to the higher toxicity of Cr(VI) compared to Cr(III). Total Cr and Cr(VI) were determined, the latter through an alkaline extraction with NaOH followed by Cr determination in the extract by electrothermal atomic absorption spectrometry. On average, Cr(VI) was found to be present at levels of about one-tenth of total Cr; however, since Cr(VI) compounds are unstable and readily reduced to Cr(III) in the presence of electron donors such as organic matter or reducing inorganic compounds, those results await confirmation from studies employing a species-selective detection method (i.e., an analytical method able to separately measure each of the extracted Cr species).

An analytical procedure to assay the quality of commercial amino acid/peptide-based metal chelates used in animal feeds, with special reference to those made from hydrolyzed proteins (HPs) as amino acid/peptide source, has been proposed by Pastore et al. [47]. When HPs are used as bioligand sources for the formation of metal chelates, the hydrolysis of the starting proteins must be as complete as possible in order to yield amino acids rather than peptides, since the stability constants of the complexes formed by the former are higher. Moreover, a large excess of HP must be present in the commercial product to keep the complex stable even at the low pH found in the first digestive tract. The ability of the HP to bind the metal and the metal/HP ratio were assessed through a multi-technique approach comprising size exclusion chromatography, amino acid and peptide HPLC analysis, acid base titrations, and especially circular dichroism [47].

The current EU legislation permits Cu, Fe, Mn, and Zn chelates from soybean protein hydrolysates as TE sources to animal feed within the same range of dietary additions as other permitted sources of the respective TEs. A recent, thorough study on metal-complexes in soybean protein hydrolysates shows a convincing approach for the characterization of coordination compounds meant for use as feed additives [48]. Water-soluble metal complexes were determined through fractionation of the extract by size exclusion chromatography (SEC) (Fig. 1) followed by the analysis of the metal-containing fraction by reversed-phase nanoHPLC with parallel ICP-MS and ES-MS/MS detection. The metal complexes were identified in the mass spectra owing to the Cu characteristic isotopic pattern (Fig. 2). The study demonstrated the feasibility of SEC-ICP-MS to produce characteristic metal (Cu, Zn, Mn, Fe) distribution patterns, which can be of interest to test batch-to-batch reproducibility and to determine the origin of the supplement, whereas ES-MS/MS allowed the identification of a number of Cu complexes

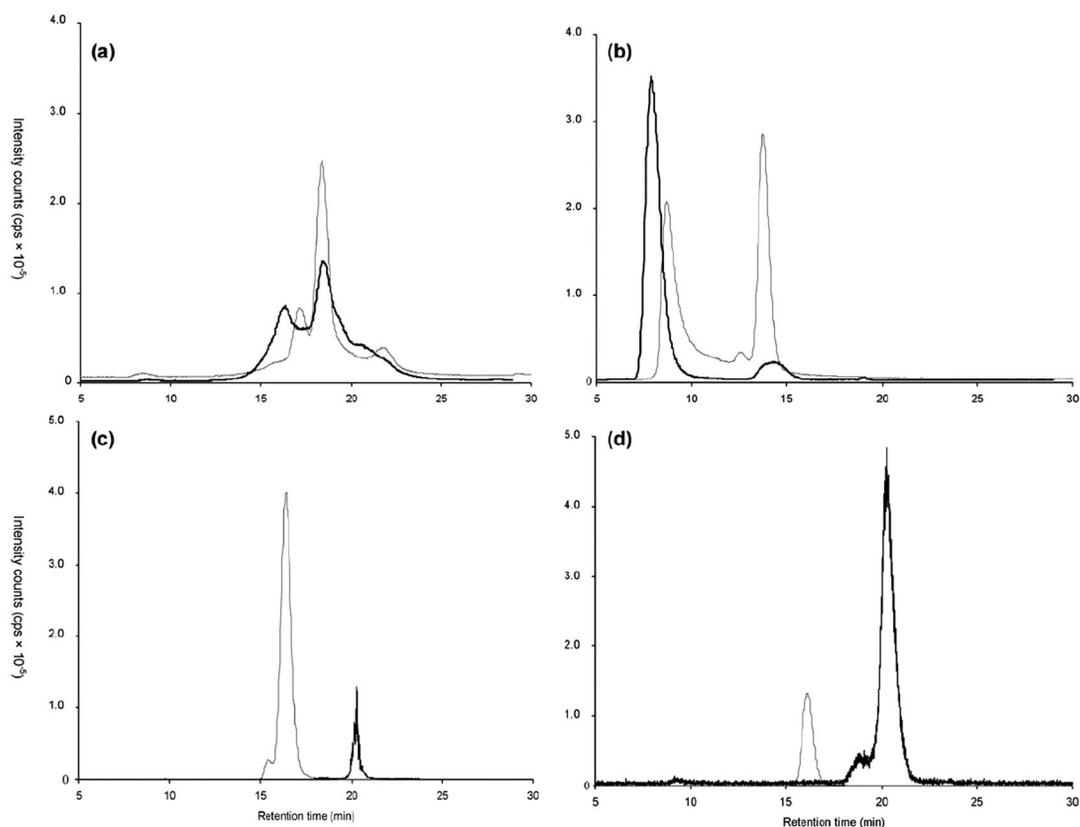


Fig. 1 SEC-HPLC-ICP-MS chromatograms of Cu (a), Fe (b), Mn (c), Zn (d) -proteinate from soybean (black) and Cu (a), Fe (b), Mn (c), Zn (d) -proteinates extracted from an alternative soybean source (gray). Reproduced from [48] by permission of The Royal Society of Chemistry.

with peptides, with different structure conformations being modeled by means of molecular mechanics investigations to assess the chelation stability.

The above examples show that promising analytical tools do exist; however, their actual applicability to real-life samples, such as premixes and feeds, needs to be demonstrated. Moreover, it may be difficult to determine labile metal complexes using chromatography coupled to mass spectrometric techniques; thus, different techniques may be needed depending on the compounds to be detected. Finally, discrimination between inorganic sources of a given TE may also be an issue in some cases, e.g., between CuO and CuSO₄ where significant differences in bioavailability do exist.

CONCLUSIONS

Organic forms of TEs, such as organic chelates, yeast-based additives, etc., appear as a cutting-edge issue for the feed industry, eager to find cost-effective sources of nutrients in order to support intensive animal husbandry. Since feed quality is of paramount importance for the wholesomeness of foods of animal origin [1], organic form of TEs are increasingly interesting also for risk assessment in the field of food safety. The key specific aspect is higher bioavailability; if actually proven, this may lead to lower supplementation levels needed in feeds as well as to increased carry-over to consumers through enhanced tissue deposition. Thus, a risk-to-benefit balance has to be kept between the two core aspects. Currently, EFSA FEEDAP has restricted the assessment of all TEs in feeds, including organic forms, to

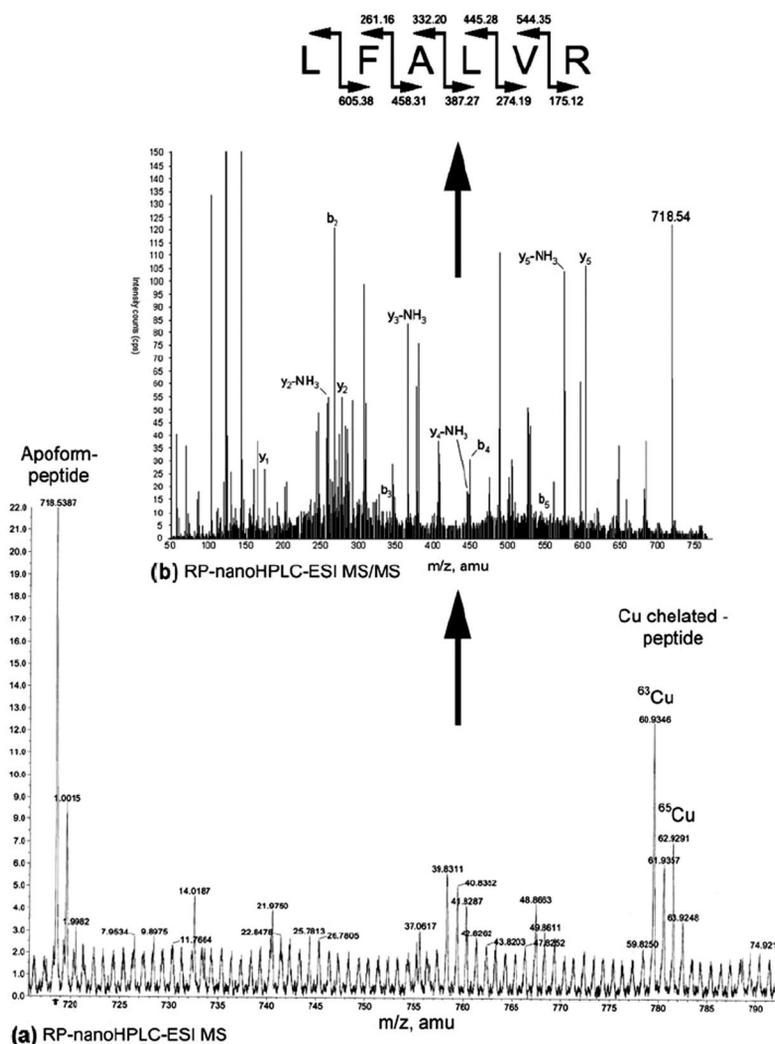


Fig. 2 (a) nanoHPLC-ESI-MS data for the ion extracted ($m/z = 718.5387$) from the Cu-protein fraction collected by preparative SEC-ICP-MS (b) MS/MS fragmentation by collision-induced dissociation of $m/z = 718.53$ species and related amino acid sequence identified. Reproduced from [48] by permission of The Royal Society of Chemistry.

their action as supplements for farm animals: nutritional additives in feeds should provide an adequate intake of bioavailable TEs in order to prevent and/or manage large-scale problems of essential elements availability for farm animals. There will be an indirect advantage for human nutrition, as adequately nourished animals will deliver products with an optimal content of nutrients. On the other hand, EFSA looks with great caution toward attempts to use TEs, as well as other nutritional feed additives, as a way to indirectly “fortify” foods of animal origin with high levels of micronutrients [23].

Until now, FEEDAP opinions indicate that the organic forms of TEs generally have somewhat higher bioavailability compared to their inorganic counterparts. Nevertheless, the margin of safety for farm animals remains essentially unchanged, and no additional or specific toxicological effects have been identified. On the other hand, the tissue deposition may change, as observed for deposition in muscle following treatment with selenized yeasts [6,10,11]. The attention toward potentially increased carry-over to consumers may, therefore, be justified; this issue is especially relevant for essential TEs

with known potential for human toxicity (e.g., Se or Mn). Therefore, tissue deposition is a key element in order to build up the assessment of consumer exposure. Consumer exposure scenarios should be based on conservative but realistic consumption figures [6] and consider the intake levels of the TE from other sources, including the background in foods of animal origin: indeed, the key element is to characterize the additional contribution resulting from the use of the TE source in feeds. In the case that ULs have not been defined, such as for Cr(III) and Mn, such contribution should not lead to a significant increase of current human intake levels [8,14]. Currently, two major refinements of consumer exposure assessment can be indicated. First, the species of the TEs involved in deposition in specific edible tissues or products should be characterized, as this may be relevant to consumer safety. One example, outside the feed field, is represented by As compounds in fish, where deposition is represented mainly by poorly bioavailable arsenobetaine [21] as well as by less known arsenolipids [49]. Another example is Se, whose metabolic fate depends on the chemical form: the inorganic Se species are directly reduced to selenide (Se^{2-} , the key intermediate of the Se metabolism), whereas selenomethionine is partly metabolized to selenocysteine and then to selenide, and partly incorporated into body proteins as Se reservoir before entering Se metabolism [6]. A second refinement concerns the availability of realistic food consumption data, including specific population groups. ULs are age-weighted; alike xenobiotics, children might be more vulnerable to excess exposure to trace nutrients simply because of higher food intake relative to body weight. Age and gender may influence the amount and type of food consumption, hence, both the exposure associated with the additive use and the background intake [50]. Assessment of age- and gender-related vulnerability and exposure is important to implement the new concept of “sustainable food safety”, where prevention of risks should be targeted also to the generation to come [51].

Overall, organic forms of TEs are a growing component of feed additives, hence of production of foods of animal origin, that require knowledge-based management. When enhanced bioavailability is supported by a robust database, it might be possible to envisage maximum content of organic forms of TEs different from those already existing for background/inorganic forms. This will require analytical methods apt to guarantee quality of the additive (i.e., in what form the TE is actually present) and to monitor feeds; thus, achieving effective management will ultimately rely on the development and transfer to control practice of specific analytical methods capable of distinguishing between organic and inorganic forms of TEs in the relevant matrices.

ACKNOWLEDGMENTS

The present paper has been performed within the activity frame of the 6th Integrated Project AQUAMAX (<<http://www.aquamaxip.org>>), as well as of the Italian Society Environment and Health-ISEH (<<http://www.iseh.it>>) and the nonlucrative organization of social utility Nutrition & Food Safety and Wholesomeness-NOODLES (<<http://www.noodlesonlus.org>>).

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