Maisons-Alfort, 28 May 2014

THE DIRECTOR GENERAL

OPINION
of the French Agency for Food, Environmental and Occupational Health & Safety

concerning a request for scientific and technical support to analyse a study published in 2013 falling in the scope of the dossier on active substances containing copper compounds in plant protection products

ANSES undertakes independent and pluralistic scientific expert assessments.
ANSES primarily ensures environmental, occupational and food safety as well as assessing the potential health risks they may entail.
It also contributes to the protection of the health and welfare of animals, the protection of plant health and the evaluation of the nutritional characteristics of food.

It provides the competent authorities with all necessary information concerning these risks as well as the requisite expertise and scientific and technical support for drafting legislative and statutory provisions and implementing risk management strategies (Article L.1313-1 of the French Public Health Code).

Its opinions are made public.
This opinion is a translation of the original French version. In the event of any discrepancy or ambiguity the French language text dated 28 May 2014 shall prevail.

On 7 November 2013, ANSES received a formal request from the Directorate General for Food (DGAL) to provide scientific and technical support to analyse a study published in 2013 showing the potential toxicity of copper in relation to Alzheimer's disease.

1. BACKGROUND AND PURPOSE OF THE REQUEST

In the framework of the European evaluation of active substances in plant protection products (Regulation (EC) No 1107/2009[1]), France is the rapporteur Member State for active substances defined as "copper compounds". Further to an assessment carried out by ANSES, the European Food Safety Authority (EFSA) published its conclusion in 2008 (EFSA 2008).

Copper compounds were included on 1 December 2009[2] in Annex I of Directive 91/414/EEC with a request for confirmatory data, for which ANSES's assessment was finalised and submitted to the European Commission during 2012. The European review by EFSA on the confirmatory data was published in 2013 (EFSA 2013).

A scientific study was published in 2013 in the PNAS journal (Singh *et al.* 2013). This article reported the results of a series of experiments aimed at determining the mechanisms of cerebral accumulation of amyloid-β induced by low oral doses of copper.

In this context, ANSES received a request from the DGAL to analyse the findings in the publication by Singh *et al.* 2013. The Agency was called on to address the following points in particular:

[1] Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC.

[2] Commission Directive 2009/37/EC of 23 April 2009 amending Council Directive 91/414/EEC to include chlormequat, copper compounds, propaquizafop, quizalofop-P, teflubenzuron and zeta-cypermethrin as active substances, OJ No L 104, 24.4.2009, p. 23-32.

- the scientific justification for a relationship between exposure to copper and copper compounds, and Alzheimer's disease;
- a comparison between copper levels used in the study and those used in toxicity studies included in the active substance dossier;
- a comparison between copper levels used in the study and those to which operators, agricultural workers, residents and consumers may be exposed.

2. ORGANISATION OF THE EXPERT APPRAISAL

This expert appraisal was carried out in accordance with the French standard NF X 50-110 "Quality in Expertise - General Requirements of Competence for Expert Appraisals (May 2003)".

The appraisal was performed by the Regulated Products Department at ANSES and the Expert Committee (CES) on "Plant protection products: chemical substances and preparations" was consulted on 25 March 2014.

3. ANALYSIS

Key points of the publication

The publication reports the results of low-dose exposure to copper administered orally to normal laboratory mice (i.e. non transgenic animals) and to a transgenic mouse model expressing a mutant human amyloid-β (Aβ) protein.

The study shows accumulation of copper in brain capillaries in normal aging mice not exposed to copper, but not in the brain parenchyma, and a reduction in levels of LRP1 protein (low density lipoprotein receptor-related protein 1) associated with increased levels of amyloid-β protein.
Chronic oral exposure to low doses of copper from 2 months of age reproduces and accelerates these effects, involving also a reduction in LRP1 in brain capillaries. *In vitro* studies support these results by reproducing similar changes in a dose-dependent manner in murine and human endothelial cells exposed to copper levels similar to normal plasma levels of labile copper. These studies also suggest that the reduction in LRP1 is at least in part related to a copper/LRP1/prion protein interaction, to its nitrotyrosination and to its proteasomal degradation.

In the transgenic mouse model overexpressing a mutant form of human amyloid-β precursor protein (APP), oral copper exposure from 6 months of age also caused an increase in copper content in brain capillaries, but also in the brain parenchyma, leading to an accumulation of Aβ associated with a decrease in LRP1 levels in endothelial cells, as well as with a state of oxidative stress in the brain and neuroinflammation.

In conclusion, these findings provide highly fundamental mechanistic data indicating that the increase in Aβ observed in the brain is related to the reduction in LRP1 protein, transporter of Aβ. They suggest that the selective action of copper on the LRP1 endocytosis receptor may contribute to a worsening or progression of Alzheimer's disease. One of the main interests of the Singh *et al.* study is the focus on a normal animal model (young or aging mouse) with exposure to low doses of copper to evaluate the effect on one of the mechanisms involved in progression of the disease. These results are supported by *in vitro* and *in vivo* analyses in a transgenic mouse model, while the difficulties of interpreting results in these types of models are highlighted.

Scientific justification for a relationship between exposure to copper and copper compounds and Alzheimer's disease

In human pathophysiology, many studies point to the role of metals in the development of human neurodegenerative diseases, and specifically the role of copper in Alzheimer's disease (Jellinger 2013). Many studies have reported an increase in labile copper, not bound to ceruloplasmin, in the most affected brain regions and the plasma (Squitti and Polimanti 2013), and its relationship to

cognitive decline especially in hyperlipidemic patients (Squitti *et al.* 2009). Amyloid plaques, one of the characteristic lesions found in the disease, contain copper but also zinc and iron. Some epidemiological studies have demonstrated accelerated cognitive decline associated with high dietary intake of copper, which is more obvious when there is also a high intake of highly saturated and trans fats (Morris *et al.* 2006). Changes in copper homeostasis and hypercholesterolaemia were therefore identified independently as synergistic risk factors for the disease (Hung *et al.* 2013).

Singh *et al.* hypothesise that reduced vascular LRP1 plays a role in accumulation of Aβ, leading to a change in passage of Aβ across the blood-brain barrier. This suggestion is not new and was suspected in findings published in 2004 (Deane *et al.* 2004).

The Singh *et al.* study looks at the amyloid-β (Aβ) protein and its role in the molecular pathophysiology of Alzheimer's disease. However, this is a complex disease that is still poorly understood. The hypothesis known as the "amyloid cascade" postulates that accumulation of Aβ in the brain, resulting from abnormal cleavage of APP, is an essential, preliminary event in Alzheimer's disease. Accumulation of Aβ in brain tissue is an important event. However, the characteristic lesions of Alzheimer's disease are made up not only of amyloid deposits, but also of neurofibrillary tangles containing tau protein. The article does not study the tau protein and addresses only one pathophysiological aspect of Alzheimer's disease.

Given this complexity, molecular pathophysiology can be examined with conventional or transgenic animal models that express one or more of the human proteins involved in the disease.
Transgenic mice can be used to model some molecular characteristics of the disorder. However, interpretation of the results must take into account the specificities of the different models. The authors themselves point out the possible limitations of transgenic models. The model used in their study overexpresses human Aβ protein, which binds to copper. In this way, copper homeostasis could be intrinsically disrupted, leading to a copper deficit in these models (Bayer *et al.* 2003; Maynard *et al.* 2002).

The effect of dietary exposure to copper must also be examined in light of other studies showing a relationship between Alzheimer's disease and exposure to other metals on the one hand, and interactions with cholesterol concentrations, on the other.
In a study using a transgenic model also expressing the mutant form of Aβ precursor (Railey *et al.* 2011), intake of zinc or iron (10 mg/L) in drinking water impaired spatial memory and learning. In these studies, the effects of zinc seem to be due to reduced copper levels and therefore to an imbalance in concentrations of these two metals, rather than a direct effect of zinc. Moreover, a memory deficit related to zinc exposure had already been described in rats (Flinn *et al.* 2005).
Other reports confirm development of a gradual imbalance in metal levels (Fe, Cu, Zn, Al) during neurodegenerative processes in relation to the decline in cognitive functions (Chacon *et al.* 2003; Gonzalez-Dominguez *et al.* 2014; White *et al.* 2004). In mice, effects on the state of oxidative and pro-inflammatory stress in the brain, and the increase in Aβ, are associated with a memory and spatial learning deficit, observed only with concomitant intake of cholesterol and copper.

Concerning interactions with cholesterol, studies have documented an accumulation of Aβ following addition of trace amounts of copper to drinking water (0.12 ppm) in rabbits that rapidly develop senile plaques when given a cholesterol-rich diet (Sparks and Schreurs 2003; Sparks *et al.* 2011). The synergistic effects of concomitant dietary intake of copper and cholesterol have also been documented in the rat (Arnal *et al.* 2013) and mouse (Lu *et al.* 2009).

Ultimately, the Singh *et al*. study targets a molecular mechanism that may contribute to the pathogenesis of Alzheimer's disease and the authors remain cautious on interpretation of these findings.
Many studies indicate a link between copper and Alzheimer's disease, but interpretation of the findings is not necessarily straightforward because of the intrinsic complexity of the pathophysiological mechanisms in the disease that are still poorly understood, and the wide range of experimental models whose individual relevance makes interpreting results difficult.

However, studies carried out using various conventional models in different species tend to indicate an effect of copper overexposure in light of changes in amyloid-β protein regulation observed in the pathophysiology of Alzheimer's disease. This relationship also appears to involve significant interactions with other exposure factors that are also possibly involved, in particular with other metals and/or cholesterol.

Comparison between copper levels used in the study and those used in toxicity studies included in the active substance dossier

The Singh *et al*. study aims to examine the molecular mechanisms of very low doses of copper on the pathophysiology of Alzheimer's disease, whereas the studies included in marketing authorisation (MA) applications use doses that are much higher, intended to identify the potential toxic effects of copper on all organs and tissues as part of hazard identification. The "observed effects" in the two cases are therefore not directly comparable.

The short and long-term studies presented in the draft assessment report enable the risk assessor to determine daily acceptable doses in humans on the basis of studies in which no toxic effect was observed in animals. Changing from one species to another requires use of conversion factors. The no observed adverse effect levels for health derived from laboratory studies validated at the European level in the framework of the monograph are summarised in Table 1 (Annex I, EFSA 2008).

Table 1: NOAEL[3] based on experimental animal studies for active substance containing copper compounds.

Short-term toxicity (90 days)	NOAEL[3] (oral)
90-day rat	16 mg Cu/kg bw[4]/day
90-day mouse	97 mg Cu/kg bw/day
1 year dog	15 mg Cu/kg bw/day
Long-term toxicity (2 years)	
2 years rat	27 mg Cu/kg bw/day

To compare exposure levels in the Singh *et al*. study (expressed as concentrations in drinking water) with those in short- and long-term studies in the copper compounds dossier (expressed in daily doses by body weight), the concentrations in the study were converted to daily doses by body weight using the mean default factors proposed by EFSA or the FDA for the various contamination vectors (diet, water), species, and duration of toxicity studies. The results are presented in Table 2.

Table 2: Conversion of copper contents in drinking water (mg/L) into daily doses (mg/kg bw/d) in mice.

$CuSO_4$ concentration = 0.13 mg/L (drinking water)	**Sub-chronic studies**	
	EFSA	**FDA**
CF*	0.15[5]	0.265[6]
Dose (mg **$CuSO_4$**/kg bw/d)	0.0195	0.0344
Dose (mg **Cu**/kg bw/d)**	0.0050	0.0088
* mean conversion factor for both sexes ** for a copper content in $CuSO_4$ of 25.4%		

[3] NOAEL: No observed adverse effect level
[4] bw: body weight
[5] Guidance on selected default values to be used by the EFSA Scientific Committee, Scientific Panels and Units in the absence of actual measured data, EFSA Journal 2012;10(3):2579
[6] Guidance for Industry Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers, U.S. Department of Health and Human Services, Food and Drug Administration, 2005

The concentration of 0.13 mg/L of $CuSO_4$ in drinking water administered to mice for 90 days, although 52 times higher than the normal value (Singh *et al.,* 2013), amounts to a copper dose of 0.005 to 0.0088 mg/kg/day.

Even though the "effects" are not directly comparable (mechanism of action *versus* adverse effect), these calculations clearly show that the Singh *et al*. study involves an exposure level that is far below (about 1000 times) that examined in the European dossier (Table 2). These results should be examined in the light of differences in sensitivity and copper regulation between species.

Comparison between copper levels used in the study and estimated exposure levels for operators, agricultural workers, residents, and consumers

Comparing estimated exposure doses in humans, whether in workers or consumers, with the doses used in the study, calls for the use of theoretical conversion factors that differ depending on the model. In addition, this comparison is only relevant if we consider that: 1/ the effects of the tested substance are identical in humans and animals (mice in this case); 2/ effect doses are similar in conditions of excess and in conditions of deficit; and 3/ copper is regulated in the body in a similar way in all the species. However, literature data indicate that the effects differ from one species to another for many substances.

1- Conversion of the doses in the study for mice into daily doses for humans.

The data in the Singh *et al*. study were converted to daily doses in humans using the table on the basis of the conversion factors recommended by EFSA or the FDA (Table 3).

Table 3: Conversion of copper contents in drinking water (mg/L) into daily doses (mg/kg bw/d) in humans.

$CuSO_4$ = 0.13 mg/L (drinking water)				
	Sub-chronic studies		**Human dose equivalents *****	
	EFSA	**FDA**	**EFSA**	**FDA**
CF*	0.15	0.265	(Weight A / Weight H)$^{1/4}$	
Dose (mg **$CuSO_4$**/kg bw/d)	0.0195	0.0344	0.0028	0.0049
Dose (mg **Cu**/kg bw/d)**	0.0050	0.0088	**0.0007**	**0.0012**
Dose (mg **Cu**/d)			**0.049**	**0.084**
* Mean conversion factor for both sexes ** For a copper content in $CuSO_4$ of 25.4% *** Dose H / Dose A = (Weight A / Weight H) x (Weight H / Weight A)$^{3/4}$ = (Weight H / Weight A)$^{-1}$ x Weight H / Weight A)$^{3/4}$ = (Weight A / Weight H)$^{1/4}$				

2- Operators, workers and residents

Preliminary comparisons can be established on the basis of ANSES's opinions for the period 2009 to 2011 concerning products containing copper currently authorised in France and for which estimates of exposure of these population groups are available (Table 4). These opinions mention the percent of acceptable operator exposure levels for copper, or "AOEL", determined to be 0.072 mg/kg bw/d when included in Annex I of Directive 91/414/EEC (EFSA 2008). The highest estimated exposure levels (expressed in % of AOEL), derived from ANSES's assessments of all copper- or copper compound-containing products, are expressed in dose by body weight and are presented in Table 4.

In this way, the direct comparison of estimated exposure levels for operators, workers and residents with dose equivalents extrapolated from the publication by Singh *et al*. is presented in Table 4 as a ratio.

Exposure through regulated uses other than plant protection products is not included in this estimate.

Table 4: Comparison of estimated exposure doses for operators, workers, and residents with the doses in the study.

	Operators	**Workers**	**Residents**
	Max	Max	Max
% AOEL	95%*	83%	55%
Uses	Peach, plum, olive, hazel, cherry, apricot trees	Grapevines	Peach, plum, olive, hazel, cherry, apricot trees
Dose mg/kg	0.0684	0.0598	0.039
Ratio of exposure dose to human equivalent dose from the study (see Table 3)			
EFSA	98	85	56
FDA	57	50	32.5

*values obtained without use of personal protection equipment (PPE)

Since the doses used in the Singh *et al*. study are very low (estimated human dose equivalent of 0.0007-0.0012 mg/kg bw/d, see Table 3), these calculations show the considerable gap between estimated exposures for these population categories and exposures in the study. Moreover, the human doses calculated on the basis of the study appear to be far lower than the no observed adverse effect levels established in toxicity studies for the European regulatory dossier.

3- Consumers

Considering the median base level of copper in food and using the PRIMo[7] model, the estimated level of exposure for the most exposed population group is 56.4% of the Acceptable Daily Intake (ADI[8]) (0.085 mg of copper/kg bw/d). This median base level takes into account dispersion of copper contamination for each food type (ubiquitous copper; use in plant protection products; use as a fertilizer; use as a food additive in livestock; use in veterinary medicinal products, and so on).

Given the uses and currently authorised doses of plant protection products containing copper, the median base copper levels in food were replaced by median copper values (supervised trials median residue values - STMRs) for the plant treated. This refined scenario considers that crops that can be treated with plant protection products containing copper are systematically treated ("worst case" exposure scenario). As such, the exposure level for the most exposed population group is 77.2% of the ADI, (i.e. 0.115 mg/kg bw/d).

Considering intake through tap water and the median concentration of copper in tap water in France of 0.028 mg Cu/L (Health & Environment Information System on Water - *SISE-eaux* database) and the amount of water consumed in Europe as evaluated by EFSA, water contributes only a small amount of copper. Exposure related to intake of water is less than 1% of the ADI, at 0.0015 mg/kg bw/d. However, if we consider the regulatory value of copper in water, i.e. 2 mg Cu/L (EU Directive 98/83[9]), copper intake through consumption of water can reach 0.1 mg/kg bw/d.

In conclusion, the exposure level in the study (estimated human dose equivalent) is lower than estimated exposure levels for operators, workers, and residents, and for consumers in the framework of use of plant protection products.

[7] EFSA (European Food Safety Authority), 2007. Reasoned opinion on the potential chronic and acute risk to consumers' health arising from proposed temporary EU MRLs according to Regulation (EC) No 396/2005 on Maximum Residue Levels of Pesticides in Food and Feed of Plant and Animal Origin. 15 March 2007.

[8] ADI of copper = 0.15 mg/kg bw/d (SANCO/150/08)

[9] European Commission (1998); Council Directive 1998/83/EC of 3 November 1998 on the quality of water intended for human consumption. Official Journal of the European Communities, 05.12.1998, L 330/32.

Comparison with copper homeostasis limits

Copper is a known essential trace element for living organisms used in multiple physiological functions. It is regulated homeostatically ensuring that biological concentrations remain within a narrow range. Outside this range, adverse effects can occur due to deficiency or excess of copper (see Figure 1).

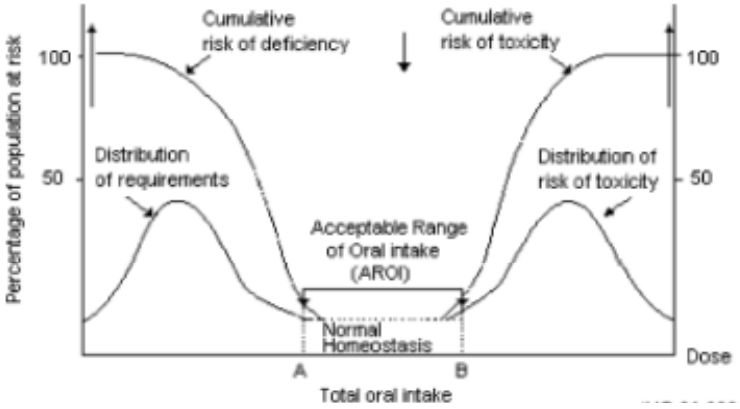

Figure 1: Theoretical representation of the acceptable range of oral intake levels of copper in humans (IPCS 2002).

The upper and lower limits of the acceptable range of oral intake (AROI) for copper were recently examined (Chambers *et al.* 2010). The authors of this publication developed a theoretical regression analysis model making it possible to use a large updated database on copper and to establish dose-response relationships for copper deficiency and excess.
This model leads to a recommended optimal intake of 2.6 mg of Cu/d in humans, a value that is higher than the Recommended Dietary Intake (RDI) of 0.9 mg/d according to the US Food and Nutrition Board (Food and Nutrition Board 2001).
The upper limit of the range, established by the US Food and Nutrition Board as 10 mg Cu/d on the basis of no change in hepatic function in adults, has also been under debate. Although the authors do not propose a new value for this limit, they point out the lack of data in humans for durations greater than 100 days and the difficulty of evaluating the impact of chronic exposure. On the basis of other research (O'Connor *et al.* 2003; Turnlund 1991), the European SCF[10] established the upper intake level at 5 mg/day (SCF 2003), considering a uncertainty factor of 2 to take into account individual variability within the normal population.

Using these correction factors, the daily dose ingested by mice in the Singh *et al*. study could be converted into a daily human dose equivalent (70 kg) of 0.05 to 0.084 mg/d, a value that is about one tenth of the RDI proposed by the US Food Nutrition Board or 100 times lower than the upper limit of the daily acceptable human consumption range.
The upper and lower oral intake limits are highly variable depending on the species: for example, these levels are low for sheep, a species that is very sensitive to copper, and high for pigs, animals that are readily deficient in copper. Determining the limits for mice would be useful to interpret the results.

[10] Scientific Committee on Food, European Commission.

4. CONCLUSIONS AND RECOMMENDATIONS OF THE AGENCY

The research carried out by Singh *et al.* 2013 concerns fundamental mechanistic studies that describe changes in the regulation of Aβ protein in the brain related to dietary exposure to copper. These changes have often been observed in the brain, in the last few years in studies carried out in several animal species.
Interpretation of these findings and of the relationship to copper should be considered in a context where data are still lacking on the pathogenesis of Alzheimer's disease which involves multiple and complex mechanisms, including the role of transition metals such as copper, iron, and zinc, as well as lipids, specifically cholesterol.

Moreover, copper is an essential trace element for living organisms. As a result, it is controlled by homeostatic regulation which maintains biological concentrations in the body within a narrow range of values.

The comparison between the copper levels used in the study and those used for the toxicity studies included in the active substance dossier shows significant differences that can be explained by the distinct objectives of each type of investigation. The study carried out by Singh *et al*. uses very low doses administered via drinking water to demonstrate a hypothesis concerning a molecular mechanism that could explain, in a specific model, the effects of copper on the pathophysiology of Alzheimer's disease. In contrast, toxicity studies included in the active substance evaluation dossier are aimed at identifying the type and extent of effects (target organs, induced disease, etc.) and establishing a dose at which no adverse effect is observed.
Calculations based on mean conversion factors proposed by EFSA or the FDA show that the study by Singh *et al*. involves a copper exposure level that is far below that examined in the European dossier for active substances containing copper compounds.

Transposition of the dose used in the publication to daily human doses, and the comparison with estimated exposure doses in humans (workers, operators, residents, consumers) causes difficulties because of sensitivity and regulation mechanisms (homeostasis) that may differ between species.

However, a preliminary comparison was carried out using risk assessments already available as part of MA applications for plant protection products containing copper and copper compounds. The results show a wide gap between the dose used in the study and the exposure doses that were considered acceptable, with respect to Regulation (EC) No 1107/2009, during risk assessment.

Moreover, on the basis of the same comparison method, the exposure level in the Singh *et al*. study is also much lower than the dietary reference intake (DRI) for copper recommended by the US Food and Nutrition Board and than the upper copper homeostasis level for humans established by the European SCF.

In the absence of data on copper homeostasis in mice and of data on the administered dose in the Singh *et al*. study on homeostatic regulation curves, the results of this study are difficult to interpret in terms of observed effects in toxicity studies carried out at much higher doses.

Marc MORTUREUX

KEY WORDS

Copper compounds, toxicity, Alzheimer's disease, operator exposure, worker exposure, resident exposure and consumer exposure.

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