ANSES Scientific & Technical Support
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The Director General

Maisons-Alfort, 2 February 2023

Scientific & Technical Support NOTE of the French Agency for Food, Environmental and Occupational Health & Safety

on the analysis of the article by Fouyet *et al.* (2022) put forward by economic actors to reject a classification rule proposed by the European Commission

On 6 July 2022, ANSES received a formal request from the Directorate General for Labour (DGT) and the Directorate General for Risk Prevention (DGPR) to provide scientific and technical support for the following: analysis of the article published by Fouyet *et al.* (2022)[1] submitted by economic actors to ANSES's supervisory ministries as a reason for rejecting a classification rule proposed by the European Commission for complex substances.

1. BACKGROUND AND PURPOSE OF THE REQUEST

Essential oils can have very complex compositions and therefore fall under the category of so-called "UVCB[2]" substances under REACH. For this type of complex substance (recently grouped under the term MOCS[3]), as for other types of substances, Regulation (EC) No 1272/2008 (CLP) on classification, labelling and packaging of substances and mixtures applies, as well as the rules for defining the hazard class for mixtures. In particular, where a UVCB contains a constituent (or a sum of constituents) presenting hazards covered by this Regulation above the classification thresholds, the UVCB should have the same classification as its constituent, even if data on the mixture show no effect. This is justified in particular by

[1] Fouyet S, Olivier E, Leproux P, Dutot M, Rat P. Evaluation of Placental Toxicity of Five Essential Oils and Their Potential Endocrine-Disrupting Effects. Curr Issues Mol Biol. 2022 Jun 28;44(7):2794-2810. doi: 10.3390/cimb44070192. PMID: 35877416; PMCID: PMC9323951.
[2] Substances of Unknown or Variable Composition, Complex Reaction Products and Biological Materials
[3] More than One Constituent Substance

the lack of sensitivity of toxicological and ecotoxicological test methods using short exposure periods for the identification of long-term effects. It is also an incentive to limit the use of tests involving animals. This last reason is also one of the major objectives of the REACH Regulation. This point, very clearly explained in the CLP Regulation, was re-emphasized in a document produced by the European Commission in November 2020[4].

Actors in the essential oils (EO) economy intend to demonstrate that this rule should not apply to essential oils, particularly for the identification of endocrine disruption (ED) properties.

This issue appears in an evolving regulatory context with the ongoing integration of the criteria for the identification of ED properties in the CLP Regulation based on the WHO definition and the prospect of further changes in the REACH Regulation.

A study co-funded by Laboratoires Léa Nature is reported in the following article: "*Evaluation of Placental Toxicity of Five Essential Oils and Their Potential Endocrine-Disrupting Effects*" (Fouyet *et al.* 2022).

In this context, on 6 July 2022, the Directorate General for Labour (DGT) and the Directorate General for Risk Prevention (DGPR) sent a formal request to ANSES in order to analyse this article and also to clarify the term "*hormonal modulator*" mentioned in this publication.

2. ORGANISATION OF THE WORK

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

The issues being appraised lie within the scope of the Expert Committee on "Chemicals covered by REACH and CLP Regulations" (Annex 1). ANSES critically analysed this article and had its analysis validated by the REACH CLP Expert Committee. In particular, two of its members reported on their analysis prior to the preparation of this note.

ANSES analyses the interests declared by the experts before they are appointed and throughout their work, in order to prevent the risk of conflicts of interest in relation to the points addressed in this note.

The experts' declarations of interest are published on the website: https://dpi.sante.gouv.fr/.

3. ANALYSIS OF THE PUBLICATION

The analysis of the publication focused on various elements, as reported in the article, in order to examine the coherence and robustness of the conclusions in view of the experimental protocol, its implementation and the analysis of the results in relation to other data available in the literature. The expert appraisal also discussed the proposed concept of "endocrine modulator".

[4] "Thought starter for discussion on the application of mixture rules for substances containing more than one constituent (including impurities) and potential impacts for REACH dossier evaluation process" discussed at the 37th Meeting of Competent Authorities for REACH and CLP, Open session, 17-18 November 2020 CA/74/2020.

1) An uncommon cellular model and a protocol still under development that limit the scope of conclusions in the absence of extensive validation and comparison:

The experimental model used (hPlacentox assay), is a test protocol under development for the study of endocrine disruption effects. This cell line was selected by the PEPPER[5] platform (Grignard *et al.*, 2022) as being of interest for the characterization of substances under the ED hazard class, but this model is still under validation as a Level 2 test (in the sense of the OECD conceptual framework according to the terms of Guidance Document 150). As a result, guarantees for key parameters such as reproducibility, sensitivity, specificity and range of applicability are not yet established. As long as the model is not validated, the robustness of the model must be demonstrated in order to be able to claim the reliability of the results obtained and the conclusions drawn from them.
Given the fact that the test is not yet validated, it would have been useful to compare the results obtained on the secretion of some hormones with existing results. However, the article does not reflect the existence of scientific literature that would allow the results obtained to be placed into context with pre-existing results, on the main constituents, for instance.

Concerning more specifically the cell line used, JEG-3, its representativeness, sensitivity, stability and other characteristics (American Type Culture Collection (ATCC HTB-36)) are not known. The JEG-3 cell line is derived from choriocarcinoma metastases adapted to passage in the hamster cheek pouch (Kohler and Bridson, 1971) leading to gene regulatory changes compared to trophoblastic cells *in vivo*. The only comparison made with other existing models shows contradictory results: *"The results we obtained with tea tree EO (no alteration of estradiol release) appear in contradiction with previous in vitro studies that demonstrated estrogenic effects of tea tree EO in MCF-7 human breast cells*". To support the results of the study, several cell lines should have been tested. Furthermore, it would have been interesting to discuss the results obtained between them in order to understand whether they could have a biological significance.

2) Methodological or traceability shortcomings for some important experimental parameters:

- no variability is indicated for the controls on the parameters tested (secretion of h-hCG, hPL, estradiol and progesterone) except for cell viability and P2X7 activation;

- the statistical power used varied between experiments. Surprisingly, in several experiments, the effect of diethylstilbestrol (DES), used as a positive control for h-hCG and hPL secretion, was statistically significant only at $p<0.1$. This shows that the system might be quite insensitive. Before drawing strong conclusions, it would have been interesting to discuss the variability of responses obtained with this positive control between these independent experiments (Figure 4 versus 5 for instance). Furthermore, in Figure 5(d), the induction of h-hCG secretion by DES is reported to be statistically significant at $p<0.01$ whereas the one induced by benzyl salicylate, apparently stronger, is statistically significant only at $p<0.1$. This suggests possible data reporting errors. For detailed analysis of the article, ANSES recommends gaining access to the raw data of these experiments.

[5] Plateforme Public/privé sur la pré-validation des méthodes d'essai sur les Perturbateurs EndocRiniens or *Public/private platform for the prevalidation of test methods for Endocrine Disruptor characterization*

- hormonal values expressed in ng/nmol/mUI for x cells (e.g. 1000) would have been useful for comparison with published data from the literature;

- no indication of the number of replicates is given for these controls. Furthermore, the concept of "*means of at least three independent experiments*" is not detailed, in particular at which level these experiments are independent (same cell line but different treatments, or different cell lines?);

- finally, some of the substances analysed were present in the essential oils at levels well below 80%, without any additional information on the other components. This induces great uncertainties on the direct extrapolation between the observations of the hazard properties on the main component versus the essential oil. For example, benzyl salicylate was present at only 2.16% in ylang-ylang essential oil. It then appears rather logical that the results obtained with the main component differ from those obtained with the EO. It was however in this case that the results obtained with the "key" component were the most similar to the EO. This questions the relevance of the model used to study the phenomenon of additivity of substances.

3) Interpretation of the findings beyond what the experiment demonstrates:

As mentioned above, it is expected that a mixture of 100 to 150 components does not give similar results to the "key" constituent when this is only present between 2% and 56%. In the absence of knowledge of the detailed composition of the mixture, no conclusion can be drawn regarding a possible divergent effect between the "key" component and the entire EO.

On the other hand, the discrepancies between the results observed with EOs containing a "key" component up to 95% and those observed with this same component deserve to be investigated. Thus the orange EO composed of 95.18% of limonene influenced the secretions of tested hormones in a different way to its main component. Orange EO did not induce estradiol secretion at the two lowest concentrations whereas limonene produced a statistically significant ($p<0.05$) 50% decrease in the production of this hormone compared to the control. A statistically significant increase in estradiol secretion was observed at the highest concentration (0.17 x 10-1%) of orange EO while limonene did not induce any estradiol secretion. It is therefore questionable to conclude on the basis of these results that "*Limonene induced an antiestrogenic effect, contrary to orange EO that induced an estrogenic effect*", these effects being variable depending on the dose tested. Neither orange EO nor limonene induced a statistically significant effect ($p<0.05$) on progesterone secretion, h-hCG or hPL, except for the highest concentration of orange EO tested which induced a statistically significant secretion of h-hCG ($p<0.1$).

Furthermore, when comparing these data with the available literature, five EOs were tested on the BeWo trophoblastic cell line in co-culture with H295R (androgen-producing adrenal cells that increase steroid hormone secretion from BeWo cells). This highly sensitive cell system showed steroid hormone secretion for two EOs and no hormone induction for the other three including orange EO (0.00005% and 0.0001%) (Yancu and Sanderson, 2019). This contradictory result was not discussed in the Fouyet publication.
Wintergreen EO, composed of 94.56% methyl salicylate, and methyl salicylate itself had no statistically significant effect on progesterone, estradiol or hPL secretion. Wintergreen EO caused a dose-dependent and statistically significant ($p<0.05$) increase in h-hCG secretion at the highest dose, whereas methyl salicylate did not cause any modulation of hormone secretion compared with the control. It therefore appears incorrect to conclude that "*methyl*

salicylate induced an antiprogestational effect, opposite to wintergreen EO, which induced a progestational effect."
A more balanced analysis of the results obtained, discussing the variability of the model, would have improved the value of this publication.

4) A surprising conclusion regarding the exclusion of the ED hazard (both on the EO and on the component) considering the experimental basis:

This paper reports that all the EOs tested or their "key" component modified the secretion of at least one major placental hormone. Therefore, the conclusion that these substances are not endocrine disruptors does not seem appropriate.

Indeed, by such a conclusion, the authors of the paper postulate that the absence of activation of the P2X7 receptor is sufficient to exclude the ED properties of the tested substances: "*P2X7 receptor activation would be a common cellular mechanism of toxicity for EDCs in placenta* [19, 20]". First of all, it is noted that the wording is cautious ("*would be*" and not affirmative), which deviates from a firm conclusion on the ED properties. In addition, this statement is not scientifically sound for the following reasons:

- the two references given (19 and 20) are two publications coming from the same research team who observed that about 10 EDs (all with a phenilic core) increased P2X7 activation in JEG-3 cells. This obviously does not allow a generalization to all EDs. Again, to be able to state that this receptor is the marker of an apical effect induced by an ED mechanism, it would have been necessary to support this with literature, or even simple additional experiments such as the use of knock-out cells for P2X7. Moreover, the results concerning the use of brilliant blue as an inhibitor of P2X7 activation by EDs (Fouyet et al., 2022) are highly questionable because brilliant blue was used at a concentration of 25 µM while its EC50 value is 10 nM (Jiang *et al*., 2000).

- the P2X7 receptor, a heterotrimeric ion channel, is a ubiquitous receptor whose biological activator is one of the purines or an ATP derivative and not a hormone. Activation of the P2X7 receptor by extracellular ATP induced cytolysis and an apoptotic process but also participated in the control of cell proliferation (Gusic *et al*., 2021; Illes *et al*., 2021). This receptor is expressed in many tissues and is involved in many signalling pathways, including those related to inflammation, but also in proliferation, cytokine secretion, protease activation, phagocytosis, autophagy, apoptosis, etc. (Fodor *et al*., 2020; Mishra *et al*., 2021; Martin *et al*., 2019; Adinolfi *et al.*, 2018).

- finally, while it is understandable given the context of this study, which focuses on the use of EO during pregnancy, to select a marker (P2X7 receptor activation) presented by the authors as being involved in placental effects, this choice does not make it an exclusive marker for the characterization of the ED hazard in the sense of the WHO definition. Indeed, this definition refers to the adverse effects on the whole body, and is not limited to the activation status of a receptor.

Moreover, it is interesting to note that this protein, which plays a role in inflammation, itself involved in allergy phenomena, was in fact activated neither by the EOs nor by the tested substances. Whereas, for example, terpineol has known allergenic effects and is banned from toys. The results obtained in the article question the sensitivity of this receptor as a marker of adverse effects in view of this inconsistency.

5) A concept of “endocrine modulator” introduced by the authors:

Finally, the authors propose to classify EOs and their "key" components as "*endocrine modulators*". This terminology appears new and undefined.

Insofar as the existence of an endocrine activity is one of the conditions for fulfilling the WHO definition, without this constituting a presumption of the ultimate classification, the identification of substances with such an endocrine activity is a filter - among others - for identifying substances of interest in order to prioritize those for which investigations of endocrine disruption should be pursued. An approach of this type was followed by ANSES in its April 2021 opinion on the development of "*a list of chemicals of interest for their potential endocrine activity*". The concept of endocrine active substances had also been developed in an EFSA opinion (EFSA, 2013).

However, in its 2021 opinion on the "*Development of a methodology to categorise potential ED substances in three categories, 'known', 'presumed' and 'suspected'*", ANSES also proposed different categories of endocrine disruptors according to the weight of the available scientific data and evidence in relation to the characterization of the hazard, as defined by the WHO criteria.

Therefore, it is feared that the introduction of the term "endocrine modulator" would create confusion with the "endocrine disruptor" hazard class, without bringing any new concept in relation to the procedures of identification and prioritization of substances of interest.

4. AGENCY CONCLUSION

From its experience in assessing the hazards of substances alone or in mixtures and from the analysis of the article, ANSES draws the following conclusions:

- it has been known for a long time that a mixture may not induce the same effect as one of its key or majority components; therefore it is not surprising to observe, in the analysed article, that the effect of an essential oil (a mixture of up to 150 substances) may differ from the effect observed with its "key" component. Nevertheless, the paper does not support any conclusion on how to account for interactions (antagonistic / neutral / additive / synergistic effect) between the constituents of EOs for different classes of effects, and particularly for the ED hazard class. As an example, solely on the basis of the effects seen in the study, these effects appeared more similar for an EO and its main constituent present at 2% in the EO, than for an EO and its main constituent present at 95% in the EO. However, the absence of information on the additional constituents makes it impossible to interpret the results obtained.

- the use of a recent experimental protocol, which is still under validation, does not bring sufficient scientific robustness to the results of the study. It is also surprising that the statistical analyses found differences with a value of $p<0.1$, which led to an overestimation of the number of results different from the control. Whatever the biological problem studied (from genotoxicity, a simple phenomenon studying the interaction of a substance with DNA, to much more complex phenomena such as endocrine disruption), it is impossible to draw conclusions on the basis of a single test performed on a single cell line.

- the results were not compared to existing results, which means that the data obtained with this model still under development cannot be confirmed. Moreover, even within the model, there was no discussion of the different results in order to assess their biological relevance. At this stage, ANSES considers that the conclusions should be considered as working hypotheses and not as firm conclusions.

- for various reasons detailed above, the reasoning given for excluding the ED properties, in the sense of the WHO definition, of both the studied essential oils and their respective "key" components does not seem sufficiently justified.

- ANSES considers that the term "endocrine modulator", which was mentioned but not defined, does not add anything new compared to the notion of substance with an endocrine activity, which is useful in a sorting and prioritization stage (EFSA 2013, ANSES 2021), and can only generate confusion in relation to a classification criterion with regard to the hazard (ANSES, 2021).

The challenge of the CLP Regulation is to propose rules that are simple and protective, that allow the classification of a very large number of mixtures and that optimize the use of animal testing. In addition, by analogy with the CMR hazard classes, the rule for mixtures makes it possible to overcome the limitations of animal testing. Although animal testing is currently the preferred model for predicting human toxicity, it has limited sensitivity for detecting certain effects because of the small number of animals and the limited duration of the experiments for chronic effects. Therefore, it would not appear proportionate, whether for the EO sector or for complex mixtures in general, to test each of the mixtures.

Thus, after analysis, ANSES considers that this paper does not constitute a solid basis for questioning the methodology currently used to classify mixtures including EOs, for the reasons developed above. While the article seems to conclude on the ED properties of five EOs and their "key" components, its limitation in observing hormonal variations and the activation of a molecular marker only allows a comparison in the context of an *in vitro* test, i.e. without integrating the response at the global level of the organs or the body.

This comparison might be interesting if further explored by comparing it with other results published in the scientific literature, particularly in order to gain validation of this Level 2 protocol for the characterization of the ED hazard. ANSES emphasizes that the complexity of the mechanisms involved more likely calls for the development of a battery of tests than one isolated test.

Finally, ANSES recalls that its previous opinions have always recommended accelerating the development of sensitive, specific and repetitive experimental methods, to allow the rapid prioritization of substances with regard to this ED hazard class, which has just been included in the latest evolution of the CLP Regulation.

Pr Benoît Vallet

KEYWORDS

Essential oils, endocrine disruption, CLP Regulation

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ANNEX 1: PRESENTATION OF THE PARTICIPANTS

PREAMBLE: The experts who are members of the Expert Committees, Working Groups or designated as rapporteurs are all appointed in a personal capacity, *intuitu personae*, and do not represent their parent organization.

EXPERT COMMITTEE

The analysis addressed in this note was conducted with the support of two experts from the Expert Committee on "Chemicals covered by REACH and CLP Regulations" (REACH CLP Expert Committee; *fourth mandate, from 1st January 2021 to 31 December 2023*)

Chair
Mr Christophe MINIER – University Professor – Université Le Havre - Normandie.

Vice-chair
Mr Fabrizio PARISELLI – Research engineer, Toxicologist – CNRS.

Members
Ms Sylvie BALTORA-ROSSET – University Professor (Université Picardie Jules Verne) – Specialities: analytical chemistry and risk assessment.

Ms Isabelle BILLAULT – University Lecturer (Université Paris Sud-Saclay) – Specialities: Analytical and Organic Chemistry, Physico-chemical properties of substances.

Mr Christophe CALVAYRAC – University Lecturer (Université de Perpignan Via Domitia) – Specialities: analytical chemistry, e-fate, biotic and abiotic degradation, microorganisms.

Mr Gwenaël CORBEL – Research fellow (CNRS) - Specialities: Synthesis and characterization of inorganic materials and nanomaterials.

Mr Richard DANIELLOU – University professor, Associate-Dean UFR (Université d'Orléans) – Specialities: biochemistry, Organic Chemistry, enzymes, cosmetics.

Mr Franck-Olivier DENAYER – University Lecturer (Université de Lille Droit et Santé) - Specialities: ecotoxicology, toxicology, endocrine disruptors, nanoparticles, metals, plants.

Ms Laure GEOFFROY – Ecotoxicologist (INERIS) - Specialities: environment, ecotoxicology, nanomaterials, endocrine disruptors.

Mr René HABERT – Distinguished University Professor (Université Paris Diderot) - Specialities: endocrinology, reproduction, development, endocrine disruptors.

Mr Philippe JUVIN – Retired - Specialities: French and European Regulations, toxicology, prevention of occupational risks.

Mr Ludovic LE HEGARAT – Deputy Head of Contaminants Toxicology Unit (Laboratoire de Fougères – ANSES) - Specialities: genotoxicity, toxicology, Toxicity Reference Values, hepatotoxicity, metabolism.

Mr Nicolas LOISEAU – Research Director (INRAE) - Specialities: chemistry, toxicology, hepatotoxicology, QSAR, pharmacology.

Mr Jean MARTINEZ – Distinguished Professor (Université de Montpellier (Faculté de Pharmacie) - Specialities: chemistry, pharmacology, endocrinology.

Mr Christophe MINIER – University Professor (Université Le Havre – Normandie) - Specialities: ecotoxicology, Regulatory context, endocrinology, endocrine disruptors.

Mr Fabrizio PARISELLI – Research engineer, Toxicologist – CNRS - Specialities: toxicology, Regulations, Occupational Health and Safety, Risk assessment.

Mr Vincent RICHARD – Safety Engineer (DIRECCTE Normandie) - Specialities: chemical hazard, Regulations, health hazards, facilities classified for protection of the environment (ICPE).

Mr Bernard SALLES – Distinguished University Professor of Toulouse University - Specialities: General Toxicology, Molecular toxicology and pharmacology, carcinogenicity, nanotoxicology, cellular models.

Ms Paule VASSEUR – Toxicology Professor, Researcher in Toxicology and ecotoxicology (Retired from Université de Lorraine) - Specialities: toxicology, alternative methods, Public Health, Health security, Environmental health, risk assessment.

Ms Catherine VIGUIE – Research Director, veterinary (INRAE) - Specialities: endocrinology, endocrine disruptors, toxicology, pharmacology.

ANSES PARTICIPATION

Scientific Coordination and scientific contribution

Ms Johanna BERNERON – Scientific Project Manager – REACH, CLP & endocrine disruptors Unit
Ms Sandrine CHARLES – Scientific Project Manager – REACH, CLP & endocrine disruptors Unit
Ms Cécile MICHEL – Head of REACH, CLP & endocrine disruptors Unit.

Administrative secretariat

Ms Patricia RAHYR – ANSES