Natural complex substances: An experimental & in silico combination approach to evaluate ecotoxicity

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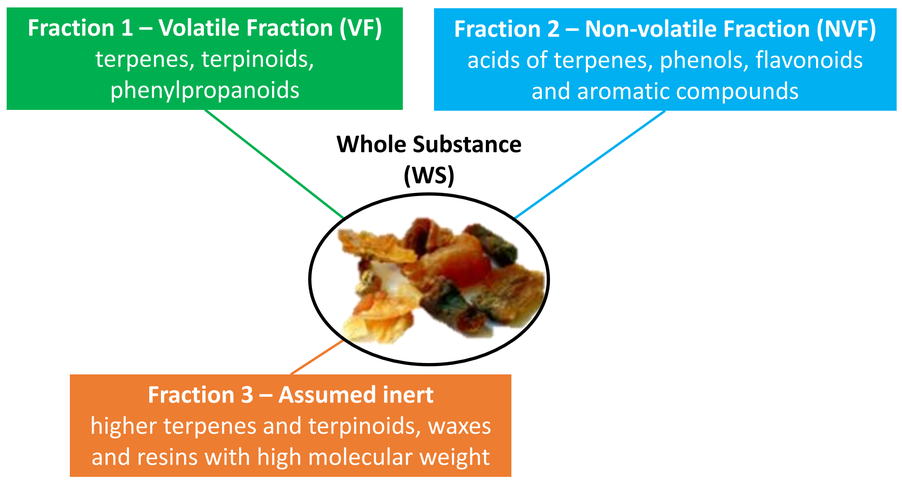
1. INTRODUCTION

Natural complex substances (NCSs) are botanical extracts of unknown or variable composition. Such NCS contain long chain substances that are very difficult or impossible to identify, representing a challenge to characterization and toxicity assessment. This study aims to provide an innovative and relevant method to **identify the constituents** of a NCS, **experimentally assess**, **accurately predict and mechanistically explain** its **ecotoxicity**.

The **CHANCES2 project** started in October 2020 with an NCS type 2, **Galbanum resinoid** (phase 1), using a discrete methodology of fractionation and ecotoxicity testing in combination with a published method for *in silico* mixture toxicity prediction. While phase 2 recently finished (**Clary Sage Concrete** (*Salvia sclarea* L.)), we are currently running the third phase in the series (**Orange oil concrete**). In this methodology we have sought the optimal strategy between ecotoxicity laboratory testing using chemical analysis and *in silico* modelling laboratory results, each approach being complementary.



2. QUESTION

Can we characterise, mechanistically understand and finally predict the ecotoxicity *in silico* of natural complex mixtures that are <50% analysable (and include 100s of constituents and resinous compounds)?

3. HYPOTHESES

The resinoid can be separated into 3 fractions then reconstituted and compared to the whole substance:

1) **Fraction 1 (VF)** known constituents: toxicity precisely **modelled *in silico*** using the iSafeRat® WAF model;
2) **Fraction 2 (NVF)** **toxicity** to organisms can be **measured** and then **modelled** (= not non-polar narcotics);
3) **Fraction 3 (inert)** is expected to be **inert** (= not to be toxic in a chronic algae & chronic daphnid limit test);

=> The results of the analytical and toxicity tests of these **three fractions** can be summed (using an *in silico* chemical activity approach) to obtain the same toxicity result as found for the whole substance (WS).

4. METHODS

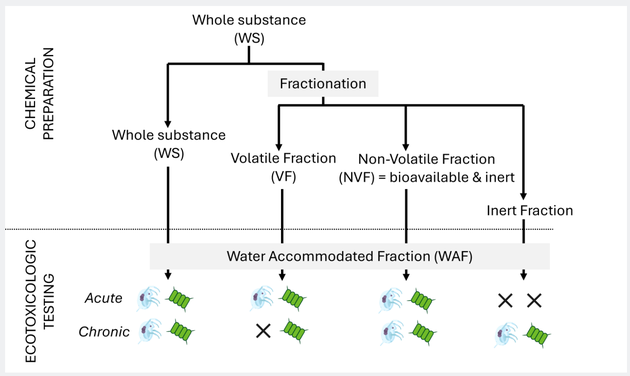
The experimental testing methodology was consistent regardless of the NCS2 tested:

1/ CHEMICAL ANALYSIS

Substances are tested whole and are split into three fractions as designated above and extensively analysed.

2/ TOXICITY TESTING

The fractions were tested following the flow chart below:



Each fraction was tested, applying the **Water Accommodated Fraction (WAF)** approach to:

- OECD 201 tests (algal 72h-EC50 and NOEC)
- OECD 202 test (daphnids 48h-EC50)
- OECD 211 test (daphnids 21d-EC10)

3/ TOXICITY MODELLING & PREDICTION

Four different approaches were tested, based on expected and adjusted fraction composition, and using WAF *in silico* approach (Bicherel & Thomas, 2021) for fractions with similar mechanisms of action (MechoA) like VF or additivity for diverging MechoAs within the same fraction, like NVF.

Predictions 1 and 3 assume that NVF et WS toxicity can be explained using non-polar narcotic MechoA, uniquely.

Predictions 2 and 4 assume that NVF et WS toxicity can be explained using additivity of different MechoAs.

Fractions	Prediction 1*	Prediction 2*	Prediction 3**	Prediction 4**
VF	WAF	WAF	WAF	WAF
NVF	WAF	Additivity	WAF	Additivity
WS	WAF	Additivity	WAF	Additivity

** According to expected composition of each fraction*

*** According to analytical monitoring during experiments*

5. NCS2 SUBSTANCE DESCRIPTION

1/ Differences and similarities in NCS2s

The 3 NCS2 differed significantly in structural diversity but also relatively between fractions:

1) **Galbanum resinoid**: Divided roughly into thirds between volatile (terpenes), non-volatile (acids…), and inert (long chain resins) fractions.
2) **Clary Sage Concrete**: The VF consisted mostly of terpenes but only at 8% of the Whole Substance. Mostly one constituent, sclareol/episclareol (>60% of the WS) dominated the NVF and the vast majority of the rest of the substance was made up of large chain acids. The inert fraction, representing approximately 10% of the whole substance, was considered to be Triacylglycerides.
3) **Orange oil concrete**: The most part of this NCS2 consists of a terpenic VF fraction which dominates the concrete.

6. RESULTS

The complete strategy for first NCS2 tested was completed and published recently (Canton et al, 2025; Thomas et al. 2025).

- For **Galbanum resinoid** It was possible to experimentally test the individual fractions (including non-toxicity of the "inert" fraction) and the whole substance adequately although the measured concentrations did not represent the nominal values (a typical problem of WAF testing).
- Despite this, the WAF *in silico* model, based on nominal/anticipated concentrations accurately predicted the ecotoxicity of the fractions of the NCS2 fractions and the WS, using non-polar narcosis MechoA.
- In experiments, analysis revealed significant differences between expected and observed concentrations (e.g., beta-pinene in WS, loss of germacrene-B, galbanic acid and farnesiferol A in NVF) -> inclusion of actual measured concentrations into the model enhanced prediction accuracy to within a factor of 2 of the experimental values using the MechoA 1.1 WAF *in silico* method.
- **Clary sage** was tested following the same strategy as the first NCS. Toxicity was observed for the VF, for sclareol tested alone and in the NVF. However, toxicity was not observed in chronic studies for the WS. No toxicity was observed for the "inert" TAG fraction.
- The *in silico* method correctly estimated toxicity for the VF and for sclareol. The other fractions are currently undergoing assessment.
- **Orange oil** is in the early stages of assessment, with identification of the constituents, fractionation approach into VF, NVF and "inert" performed and range finding studies ongoing.

7. CONCLUSION

The application of a fractionation approach in combination with an *in silico* model for mixture toxicity testing reduces uncertainty in aquatic hazard testing. The 2 approaches are highly complementary: 1/ the laboratory tests provide empirical values of acute and chronic toxicity for each fraction and the WS and validate the outcome of the *in silico* model; 2/ the *in silico* model provides a mechanistic explanation of the observed toxicity. The model can be used to predict toxicity of different constituent blends.

The use of extremely similar methodologies for the 2 other NCS and encouraging initial results on observed and predicted toxicity highlights this approach can provide a more mechanistic and comprehensive methodology to identify hazard of highly complex UVCBs.

References

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