Chemometrics and Intelligent Laboratory Systems 159 (2016) 58–68

Contents lists available at ScienceDirect

Chemometrics and Intelligent Laboratory Systems

journal homepage: www.elsevier.com/locate/chemolab

Exploring the disruptive effects of TBT on lipid homeostasis of *Daphnia magna* using chemometric methods

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ARTICLE INFO

Article history:
Received 10 March 2016
Received in revised form
25 July 2016
Accepted 21 August 2016
Available online 27 August 2016

Keywords:
ASCA
MCR-ALS
Lipidomics
Daphnia magna
Ecotoxicology
Xenobiotic

ABSTRACT

This study explores the effect of tributyltin (TBT) treatment on lipid homeostasis of *D. magna* using chemometric methods. The experimental design included two factors 'time' and 'dose' of TBT, and lipids were measured in replicate samples for each combination of factors. This study represents a case of multivariate multi-set data, obtained using a two factorial balanced design and explored using different multivariate chemometric techniques, to have an insight into data patterns, to separate main sources of variance and to identify lipid profiles associated with TBT dose. ANOVA- Simultaneous Component Analysis (ASCA) allowed the separation of the two sources of variation, suggesting thatthe TBT dose affected the evolution of the concentrations of lipids over time, although when the whole development process was considered, the interaction between time and dose factors was rather low. Multivariate Curve Resolution Alternating Least Squares (MCR-ALS) helped to resolve four distinct profiles associated with the different phases of *D. magna* development, showing changes in their lipids composition, and in their relation with TBT dose. A discussion of the meaning of these changes on lipid profiles and of their time evolution is performed.

1. Introduction

In addition to the existing toxic substances, a large number of new compounds (biocides, pesticides, metallic alloys, pharmaceutical etc.) enter the market every year and subsequently find their way into the environment. These substances enter ecosystems by many pathways, including industrial discharges and leakage, municipal waste, run-off from agricultural and forestry applications, and accidents [1,2]. Among the known toxic compounds, organotin compounds (OTCs) are organometallic compounds known to be persistent, toxic, and bio-accumulative in nature, and are able to travel long distances through different media [3,4]. OTCs comprise a group of organometallic moieties characterized by an atom of tin (Sn) covalently bonded to one or more organic chains and another functional groups, such as chloride, oxide, or hydroxide, which are represented by methyl, butyl, octyl, and phenyltin groups [5,6]. Since the late 1960s, OTCs have been extensively used across the world as biocides in antifouling paints, applied on ship hulls and fishing nets, and as fungicides in agricultural crops [7]. Their release into the environment combined with their low solubility in water and high octanol–water partition coefficient has resulted in worldwide contamination of the aquatic environment [8]. Despite their gradual removal from the market and their prohibition of use, OTCs have been still detected in various environments in recent years [9,10]. Among the most toxic OTCs, tributyltin (TBT) and tryphenyltin (TPT) are well known to have the main biological impact on the hormonal asset [5], where they act as endocrine-disrupting chemicals (EDC) [11]. Endocrine perturbations, often associated with wide spread metabolic shifts, span from the aquatic species, deeply studied for their impressive biological and ecological impact, to terrestrial organisms [11].

To ascertain the health risk associated with toxic chemicals or compounds, usually, the laboratory based bioassays or toxicological studies are carried out to assess the interaction effects of individual compounds or mixtures on the living organisms. The toxicity of any compound is related to its concentration, exposure time, bioavailability, biota sensitivity and as well as the presence of various compounds in the environment. Therefore, the toxicity assays for any compound are designed to include more than one factor (like concentration, exposure time, and physical factors) in a single experiment, and require more than one experiment related

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http://dx.doi.org/10.1016/j.chemolab.2016.08.010
0169-7439/

to different treatment groups and control group, thus generating multivariate and multi-set (or experiment) datasets. Further, these kinds of datasets have different sources of variation and require efficient data analysis methods taking into account the experimental design and relationship between the different variable to understand the system underlying the variation in the data [12].

Datasets from experimental designs are usually analyzed with univariate analysis of variance (ANOVA) to estimate the significant changes in the variables or toxicity parameters in a group as compared to another control group focusing on the separation of the different sources of variability. Being a univariate method, ANOVA is not able to take the covariance between different variables into account. PCA, is based on a bilinear model which provides an abstract decomposition of experimental data which maximizes the explained variance under the constraint of orthonormality of the components [13], and SCA can be regarded as a simultaneous PCA for multiple matrices [14]. The above methods do not take the experimental design into account and the different contributions to the variation caused by the experimental design are confounded in the model hampering the interpretation of principal components [12]. The generalization of ANOVA to multiple variables: multivariate-ANOVA (MANOVA) [15] is used for the analysis of multivariate datasets with an experimental design. However, when the number of variables exceeds the number of samples, MANOVA breaks down owing to problems of singularity of covariance matrices and assumptions that are not fulfilled [16]. Recently developed method ASCA (ANOVA-based extensions of SCA) can analyze complex multivariate datasets where time is one of the factors of the data design [17]. With this method it is possible to isolate the variation in the data induced by a factor which is varied in the experimental design revealing the relation between the samples and metabolic profiles [18,19] or variables. Several variants of this approach, such as Scaled-to-Maximum, Aligned, and Reduced Trajectories (SMART) [20], Principal Response Curves (PRC) [21], ANOVA-TP [22], ANOVA-PCA [23], and regularized MANOVA (rMANOVA) [24] have been proposed.

If the dataset is composed of more than one dataset (like in toxicological studies corresponding to different conditions and factors), the extraction of practically reliable information can be improved significantly by using the methods such as multivariate curve resolution methods, benefitting from richer information contained in the data structures (like multi-way data sets) [25,26]. Multivariate Curve Resolution-Alternating Least Squares (MCR-ALS) is a flexible multivariate data analysis method which focus on describing the evolution of the experimental multivariate measurements through their underlying individual component contributions, without imposing hard-to-accomplish constraints from a natural (chemical, physical or biological) point of view, as orthogonality in the components [27]. Ambiguities associated with bilinear models, such as those occurring with the application of MCR-ALS, can be reduced by applying appropriate constraints in the MCR model during resolution [28–30]. MCR-ALS has been used to analyse different type of problems and datasets (which can be described by a bilinear model) related to many kind of processes and mixtures such as chemical reactions, industrial processes, chromatographic analysis, spectroscopic and hyper-spectral measurements, environmental data, and environmental monitoring, among others [31]. If the observed changes in the datasets under study is considered to be the effect of a combination or of a mixture of common underlying changes (patterns, sources) in the levels of different variables (also for toxicological datasets), multivariate resolution methods, like MCR-ALS can provide the opportunity to analyse them in a more comprehensive and natural manner, by resolving complex mixtures of effects into pure-components contributions in situations with no or little prior information available.

The water flea, Daphnia magna, is an OECD test species and is utilised internationally for toxicity testing to screen the toxicity of chemicals, enabling rapid and accurate categorisation into classes of defined mode-of-action, and prioritising chemicals for further testing [32]. In this work applicability of different multivariate data analysis methods to analyze datasets generated from biological/toxicological analytical studies is explored. The dataset under study is related to the experiments conducted on *Daphnia magna* providing a typical example of toxicological analytical studies. The overall objective of this study is to analyse the disruptive effects of TBT on lipid homeostasis in *Daphnia magna*, using analytical (LC-MS) and multivariate chemometric methods to (i) assess the effect of different factors of the experimental design on the overall variance present in the dataset, and (ii) to identify the main profiles associated with changes in lipids concentrations in *D. magna* with time and TBT treatment.

2. Materials and methods

2.1. Experimental design and data acquisition

The experiments were performed to study effects of TBT treatment [TBT doses: 0.1 μg/L (L), and 1.0 μg/L (H)] on the dynamics of lipids across an entire adolescent inter-molt cycle of *Daphnia magna* (Clone F). Experiments were conducted at high food levels ($5x10^5$ cells/mL of *Chlorella vulgaris*) and included five samplings: 0 h (just after the third molt), 8 h, 16 h, 24 h, 48 h (just after the fourth molt), and Eggs. The egg samples were obtained by debrooding the females at 48 h. At each sampling three replicates of 5 individuals were collected and processed for total lipid analysis. Due to the large number of synchronized animals needed, two different independent but consecutive experiments were performed. The experimental design used in this study can be explained by a general scheme as in Fig. 1. This work is a part of a larger study and further details about the experiments and analysis can be found elsewhere [33].

2.2. Lipids analysis

Lipidomic analysis was performed as described, with minor modifications [34]. Each replicate consisted of a pool of 5 animals that were homogenized in 500 μl phosphate buffered saline (PBS) pH 7.4 with 2,6-di-tert-butyl-4- methylphenol (BHT) 0.01%, as an antioxidant. Lipid extraction was performed by a modification of the Folch's method [35]. The liquid chromatography-mass spectrometer consisted of a Waters Aquity UPLC system connected to a Waters LCT Premier Orthogonal Accelerated Time of Flight Mass Spectrometer (Waters, Millford, MA), operated in positive and negative electrospray ionization mode. Full scan spectra from 50 to

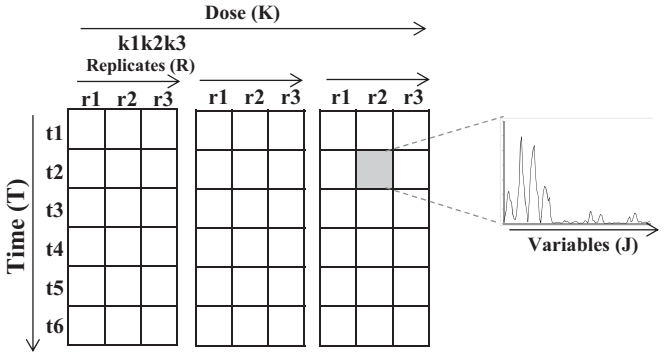

Fig. 1. Experimental design used in the study. Each square presents a sample in the design, and the corresponding lipids profile.