Metabolomics and bioanalysis of terpenoid derived secondary metabolites

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Chapter 2

Metabolomics as a bioanalytical tool for characterization of medicinal plants and their phytomedical preparations

Introduction

For many centuries, humankind used medicinal plants directly or extracts thereof for their basic healthcare needs. Medicinal plants contain a complex mixture of secondary natural products and show synergistic effects against a broad variety of diseases. According to the World Health Organization (WHO), more than 80 per cent of the world population uses medicinal plant for everyday healthcare [13]. Now there are some 50,000 different medicinal plant species used for medication and in Europe alone are around 1,300 medicinal plants commercially used [14]. In the United Kingdom more than 25 per cent of the population uses medicinal plant regularly [14], and in Germany approx. 30 % of all over the counter drugs (OTC) drugs are herbal medicinal products (HMPs).

Commercial medicinal plants in the world are mostly harvested from the wild. Not only in so called low income countries that have to face this situation, but also in Europe 90 % of medicinal plants used for extraction are collected from the wild [14]. The use of HMPs is getting more and more popular and the demographic development in Europe, USA and later in Asia [15, 16] will increase significantly the demand in the future. From these backgrounds, plant collection from the wild should be replaced by controlled cultivation to ensure sustainability of HMPs. Moreover, emerging of new illnesses, and an increase of resistance to current drugs, have emerged challenges for medicinal plant sciences as well.

HMPs are made of complex biological matrices. To ensure patient safety and a high level of quality with regard to composition and activity, sophisticated analytical methods have to be developed and applied. Production and quality management is regulated by Good Manufacturing Practice (GMP) regulations and dominantly Gas Chromatography (GC), High Performance Liquid Chromatography (HPLC), Nuclear Magnetic Resonance (NMR), Gas Chromatography Mass Spectrometry (GC-MS) and Liquid Chromatography Mass Spectrometry (LC-MS) have been introduced.

Actual developments in analytical chemistry, bioinformatics and computers have speeded up the procedures and a new strategy has been developed to get a huge pile of information from some simple testing, namely metabolomics. It is a breakthrough approach to accelerate and streamline the analytical process of medicinal plant researches. Metabolomics allows quick and efficient identification and quantification of the secondary metabolites within plants and is easily coupled to high throughput bioactivity screening. Furthermore metabolomics is well known as the youngest “omics” method. A summary of some definitions related to metabolomics is described in Table 1.
In this chapter we review bio-analytical tools often used in metabolomics of medicinal plant researches, such as NMR, Direct Injection Mass Spectrometry (DIMS), LC-MS, and GC-MS with special focus for quality control and metabolic profiling for herbal medicinal products. We highlight how bio-analytical tools are used and maybe applied in industrial routine work.

Table 1. Definitions important for metabolomics.

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<th>Metabolomics</th>
<th>• Holistic, simultaneous and systematic qualitative and quantitative determination of metabolites over time after stimulus</th>
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| Metabolom    | • Dynamic situation  
|              | • Total number of all low molecular metabolites |
| Metabolite   | • Intermediates and products of metabolism  
|              | • Primary and secondary natural metabolites (e.g. lipids, sugars, alkaloids, flavonoids)  
|              | • Low molecular compounds (mw < 1000) |
| Metabolic profiling | • Analyzes a selected group of compounds or set of metabolites in a specific biochemical pathway  
|              | • Targeted metabolites analysis |
| Metabolic fingerprinting | • Global screening approach  
| | • Classify samples based on metabolite patterns or “fingerprints”  
| | • Detect discriminating metabolites without identifying all of the compounds present |

**Bioanalytical tools**

The main purpose of metabolomics is to analyze all metabolites both qualitatively and quantitatively in medicinal plant samples. Basically metabolomics analysis consists of three steps, namely sample preparation including extraction process; metabolite measurements using bioanalytical tools, such as NMR, LC-MS, GC-MS; and mining the raw complex data.
using chemometric software. An illustration of the steps required in a metabolomic analysis of medicinal plants is explained in Figure 1.

![Workflow of metabolomics analysis in medicinal plant researches.](image)

**Figure 1.** Workflow of metabolomics analysis in medicinal plant researches.

**Sample preparation**

Sample preparation is considered as one of the most important steps in metabolomics analysis. The procedure must be standardized; starting from harvesting, extraction, storage and applying validated analytical parameters. Standardization and validation procedures are essential to receive reproducible results over time.
In harvesting, it is critical that metabolism process in plants should be stopped immediately. Damaging by cutting plants could release active enzymes (e.g. glycosidases) that can change the metabolite profile significantly. Typical degradation reactions after harvesting are oxidation, hydrolysis, and decarboxylation. To prevent those, harvesting should be done rapidly and the harvested material should be dried to stop further metabolism. Alternatively lab samples can be frozen immediately (-20°C or -80°C) or submerged in a liquid nitrogen tank and followed by mechanical disruption to release metabolites from the cells [17]. However, for commercial preparation this technique is difficult to be implemented.

First step of extraction of medicinal plants can be classified as a solid-liquid extraction. It means metabolites from a plant sample as the solid phase to transfer into the liquid extraction medium. Generally used solvents for extraction can be divided into four classes: polar solvents (e.g. methanol, ethanol, and water), medium-polar solvents (e.g. chloroform, dichloromethane, diethyl ether), non-polar solvents (e.g. n-hexane), and combined solvents thereof [17]. Each solvent will give different profiles of metabolites extracted. Metabolites that have high polarity mostly are extracted in polar solvents, while those that have low polarity mostly are extracted in medium-polar solvents. We have to consider that for commercial HMPs ethanol with and without water is the standard solvent for extraction. It seems that by one unique solvent metabolomic analysis is getting more simple, but different water: ethanol ratios and different extraction processes varying in time and temperature give a high diversity of extracts which are reflected by a high number of different HMPs on the market.

The goal of metabolomics is to identify and quantify metabolites in the biological samples consisting of a complex spectrum of different natural product classes. Currently there is no single solvent that can be used to extract all compounds directly. However, solvents which can dissolve most diverse group of compounds must be chosen, but due to discussed limitations several extractions with different solvents can be conducted to have a total view of the metabolites [18]. In the case of metabolic profiling studies with the purpose to quantify a selected group of metabolites in medicinal plants, selection of solvents should be based on the physico-chemical properties of target metabolites to be analyzed.

For NMR-based metabolomics, polar solvents such as methanol and combined solvents such as water: methanol are often used for the extractions. A two-phase solvent system, composed of a mixture of chloroform, methanol and water (2:1:1, v/v), also has been used successfully for the extractions in NMR-based metabolomics [19-21]. Moreover, the use of deuterated NMR solvents for the extraction also has been reported [22-24]. This method avoids the need
to evaporate the original extraction solvent and to redissolve the sample in the NMR solvents [18]. In LC-MS-based metabolomics samples must be dissolved in solvents preferable similar to the eluent of the HPLC system. Regarding GC-MS-based metabolomics, compounds must be volatile compounds to be measured, thus for non-volatile compounds derivatisation before measurements is needed.

**NMR:**

NMR spectroscopy is based on magnetic nuclei resonance in a strong magnetic field to determine physical and chemical properties of molecules. NMR spectroscopy basically consists of a magnet, radio-frequency (rf) transmitter or oscillator, and a suitable rf detector [25]. If an organic compound or an extract is placed in a magnetic field, interactions between NMR active nuclei, such as $^1$H and $^{13}$C, and electromagnetic radiation will produce resonance signals to be collected by the detector. Resonance frequency of NMR active nuclei is dependent on the chemical environment. Different chemical environment will give different resonances, thus each compound will possess specific NMR spectra.

NMR is widely used as bioanalytical tools for the analysis of organic molecules and considered as one of the most promising metabolomic tool [26]. It is well known as a powerful technique for elucidation of compound structures, including stereochemistry in details. NMR is non-destructive and can be used for structural analysis of metabolites in crude extracts, cell suspensions, intact tissues or whole plants [27, 28]. Moreover NMR allows the exploration of metabolic pathways, leading to qualitative information on the link between labeled precursors and their products and quantitative information on metabolic fluxes [29-32].

For NMR the $^1$H-NMR is the most popular technique used for qualitative and quantitative metabolomics analysis. It is very fast in its measurement, typically less than 5 minutes for one measurement (depending on the concentrations and the resonance frequency), facilitates high-throughput analysis and mostly has a simple sample preparation. Moreover, quantitative analysis using $^1$H-NMR has no need for external calibration curves for multiple individual structures because after normalization for the internal standard (IS) the molar concentration of a compound is directly represented by the intensity of the IS proton signal [17]. By adding an internal standard to the sample, we can compare proton signals of the internal standard with those of the sample and thus we can quantify compounds in the sample. For $^1$H-NMR, the concentration threshold for a routine detection of a metabolite in an extract using a modern high field spectrometer is probably 10 μM, corresponding to a quantity of 5 nmol in the
typical sample volume of 500 μl [30]. Furthermore, metabolomics based on ¹H-NMR approaches is highly reproducible, it means that NMR-metabolomics data are valid for ever, as long as the same extraction procedures and the same NMR-solvents are used [33].

Although the sensitivity of ¹H-NMR is low, this weakness can be solved by various approaches. Today, NMR microprobes are available, which can be used for measuring samples with low amounts of compounds. With this probe, the concentrated sample is diluted with a small volume of NMR solvent, thus it will make the sample more concentrated and give spectra with improved quality. High-resolution NMR is also available. It has high sensitivity and can give a substantial improvement in the detection of the signals, thus it produces high quality NMR spectra as well. The last approach is cryogenic NMR probes. This probe is small (3 or 5 mm in diameter) but has a capability for improving sensitivity and reducing noises with cooling the receiver coil and preamplifiers to cryogenic temperatures. It is a powerful probe that can be used for measuring small sample amounts, as has been demonstrated by Schneider et al. [34].

Another problem of using ¹H-NMR in metabolomics studies for medicinal plants is signal overlap that can obstruct the identification and quantification of metabolites. Nevertheless 2D-NMR techniques can be used to solve this problem. These techniques give better signal resolution and reduce signal overlap by distribution of the resonances in a second dimension. Moreover 2D-NMR has all advantages of ¹H-NMR but it consumes longer recording time. ¹H-J-resolved NMR (JRES) is one of 2D-NMR techniques that mostly used in metabolomics studies. JRES has capability to split the effects of chemical shift and J-coupling into two independent dimensions. An in detail review on JRES in metabolomics was reported by Ludwig et al. [35]. Beside JRES, other 2D-NMR techniques such as HSQC (heteronuclear single quantum coherence), COSY (correlation spectroscopy), TOCSY (total correlation spectroscopy), HMBC (heteronuclear multiple quantum coherence) and NOESY (nuclear overhauser effect spectroscopy) have been applied in plant metabolomics studies [28, 36, 37].

Mass Spectrometry (MS):
MS is an analytical instrument measuring the mass-to-charge (m/z) ratio of ions. MS instruments consist of three main parts, namely an ionization chamber where the molecules are being ionized, a mass analyzer which separates ions according to their m/z by applying electromagnetic fields, and a detector to record m/z. The common techniques used as ionization source are electron ionization (EI), chemical ionization (CI), electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). Meanwhile, mass
analyzers such as single quadrupole, triple quadrupole, ion trap and time-of-flight (TOF) analyzer are usually applied in metabolomics analysis.

Besides NMR, MS is well known as an analytical tool for metabolomic analysis, particularly in metabolic profiling and metabolic fingerprinting. In metabolomics, MS separates metabolites based on $m/z$ ratios of their ions. Furthermore MS also provides both sensitive detection and metabolite identification through specific fragmentation of the molecule and comparison or molecular formula determination via accurate mass measurements [26].

Moreover MS is considered as the most sensitive method for metabolomics analysis [17], because it can identify ionized components at very low quantities. A major disadvantage of using MS is its reproducibility. MS measurements are dependent on the type of MS, operating parameters and matrix effect on ionization, making it difficult to produce similar results across laboratories [17].

**Direct injection mass spectrometry (DIMS):**

A DIMS analyses metabolites with injection of a sample directly into the ionization chamber without prior chromatographic separation and provides fast and high throughput measurements. DIMS mostly use ESI and APCI as ionization sources. Both are known as soft ionizations which provide minimal fragmentation of molecular ions and a less complex mass spectrum. Therefore, with interpreting molecular ions, metabolites can be identified without chromatographic separations [38].

TOF is usually used as a mass analyzer in DIMS method. TOF separates ions based on the time of ions to reach the detector. All ions in TOF are accelerated by an electric field that makes ions have same kinetic energy. Thus the velocity of ions just depends on their $m/z$ and heavier ions will reach the detector later compared to lighter ions. Depending on the flight-tube geometry and instrument tuning, TOF-MS instruments provide mass of 6,000-17,000 with mass accuracy in the range of 3-5 amu [39]. In addition, to improve performance work of TOF-MS, a quadrupole has been attached as a scanning device or a mass filter [40]. The quadrupole has four rods with high voltage to create a quadrupole field to select ions according to their $m/z$ (only $m/z$ within a certain range can pass the rods). Therefore the quadrupole enhances capability of TOF-MS in separation of metabolites.

Different metabolites with same molecular weights cannot be separated with the previous techniques. However, the problem can be addressed by using tandem MS/MS. This technique provides great selectivity through the specific fragmentation of each metabolite. After ions (precursors) pass through the first MS, they are activated by collision with an inert gas such as
nitrogen or argon to produce ion fragments. The newly created fragment ions can subsequently be analyzed by the second MS. MS/MS is usually coupled with a quadrupole as the scanning device and TOF as the mass analyzer. The quadrupole-TOF-MS/MS instrument can clarify fragmentation process by distinguishing m/z of precursor and fragment ions. Therefore it allows high interpretation of spectra [39]. Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) is a powerful MS. It is provided with high resolution to separate and distinguish very complex mixtures and has high mass accuracy allowing calculation of elemental compositions to aid in structural differentiation and characterization [41]. Nevertheless it cannot be used to separate structural isomers. Furthermore FTICR-MS is an expensive instrument, thus the utilization is not widespread.

Gas chromatography mass spectrometry (GC-MS):
GC-MS is well known as one of the popular techniques for global metabolic profiling [42]. It provides fast analysis at relatively lower costs compared to other mass spectrometry techniques, while remaining the ability to specific metabolite detection and quantification. Furthermore GC-MS can be used to separate large number of compounds in a single measurement and identify unknown metabolites as well. GC-MS is a combined technique of gas chromatography (GC) and mass spectrometry (MS) to analyze different metabolites within a measurement. In this technique, the GC separates the metabolites while MS functions as metabolite detection tool. The GC is equipped with a capillary column as the stationary phase, a carrier gas as a mobile phase (He, N\textsubscript{2}, H\textsubscript{2}) and a sample injector. Electron impact (EI), known as hard ionization, is mostly used for ionization in GC-MS-based metabolomics. In EI, electrons are produced through thermionic emission interacting with the molecules in the gas phase to form molecular ions and fragment ions. TOF provides fast metabolite detection and can be used as mass detection, meanwhile single quadrupole and ion trap also has been used as mass analyzer in GC-MS. Metabolites identification in GC-MS analysis is conducted by comparing retention time or retention index of metabolites sample with retention time or retention index of pure reference metabolites or spectral library database [43]. Quantitative metabolomics using GC-MS requires making calibration curves of each metabolite, because sensitivity of GC-MS varies for all metabolites [17]. GC-MS only can be used for analyzing volatile metabolites with thermal stability. Derivatization can be used to measure none volatile and polar metabolites and increases volatility, thermal stability and reduce the polarities of the functional groups of metabolites.
Silylation is a derivatization procedure mostly applied by replacing active hydrogen with trimethylsilyl (TMS) group. Silylation reagent is moisture sensitive and reacts readily with water thus decreasing its efficiency. All samples should be as dry and solvents should be as clean (highest grade) as possible, to avoid inefficient derivatization. Alternatively, alkylation and acylation can be used for derivatization of functional groups, such as –COOH, -OH, -NH, and –SH.

**Liquid chromatography mass spectrometry (LC-MS):**

LC-MS is the other widely used technique for targeted or non-targeted metabolomics analysis and offers high selectivity and good sensitivity analysis. A great number of metabolites can be separated with LC-MS, since overlapping peaks of different metabolites on the chromatogram can be identified as separate compounds in the mass analyzer of LC-MS. However LC-MS has disadvantages in reproducibility of separations that can be caused by matrix effects of the complex sample extract and different parameters in LC-MS systems [17].

LC-MS instruments consist of two main parts, namely liquid chromatography for metabolites separation and MS for detection. ESI is commonly used for ionization of molecules in LC-MS, although APCI also has been used to ionize more difficult metabolites. In ESI, the sample in a suitable solvent at atmospheric pressure is ionized by application of a high electric charge to the sample needle [44]. Moreover ESI can cover a broad range of metabolites, since it operates ionization in negative and positive modes. LC-MS commonly applies TOF and single quadrupole as mass analyzer and a combination of both has been used as well. In LC-MS analysis each metabolite has a different sensitivity, therefore calibration curves of each metabolite are definitely needed for quantitative analysis.

Reversed phase column is mostly employed in metabolomics analysis using LC-MS, since it is easy to use and can be applied for separating the majority of metabolites. The characteristics of LC column are such as column internal diameter and packing particle size of column influences the level of LC-MS resolution. Improving chromatographic resolution can be achieved by reducing diameter particle of column packing material as has been applied in ultrahigh pressure liquid chromatography mass spectrometry (UPLC-MS) system [45]. This technique can reduce ionization suppression significantly and decrease co-elution of metabolites. High polar metabolites are mostly difficult separate by reversed phase columns. Alternatively, hydrophilic interaction chromatography (HILIC) columns can be used for
separation of high polar metabolites. Moreover, HILIC-MS also has been used for analysis of highly polar compounds in *Curcubita maxima* leaves [46].

**Data processing:**
The data obtained from medicinal plant metabolomics experiments are very large numbers and complex, thus very difficult to be interpreted by eye. However, after development of computer technologies and emerging of analytical software, the large amounts of data sets can be automatically visualized and interpreted. The common method for data processing in metabolomics is multivariate data analysis. It is a chemometric method that can visualize large number of compounds resulted from metabolomics experiments and data mine information about the relationships between levels of different metabolites [47]. To provide appropriate data for multivariate data analysis, the spectra from metabolomics experiments must be extracted.

Today, three methods have been developed for the raw data extraction, namely binning, peak picking and deconvolution [48]. Binning or bucketing is the most used method for data extraction in metabolomics [49]. By this method, spectra are subdivided into several regions, called “bins” or “buckets” and the total area within each bucket is used as a representation of the original spectra [50]. In NMR-based plant metabolomics, binning can reduce 16k data points to 250 data points [51] while in MS-based plant metabolomics the number of bins is usually below 2000 at a bin size of 1 amu [39]. However, binning in crowded spectra has the potential for significant loss of information, for example by including peaks belonging to multiple compounds within a single bin [52]. Peak picking method as an alternative of bucketing basically consists of peak finding, baseline subtraction and alignment steps [48]. An investigation has been conducted to compare peak picking with bucketing on the data extraction, and the results showed that the peak picking approach was more interpretable than the bucketing [53]. In deconvolution, defined as targeted profiling, the data is integrated by an algorithm from pure compound spectra and interrogated for identifying and quantifying the metabolites in the mixture [54]. In NMR-based metabolomics, this method provides NMR signal vectors and quantitative metabolite data [48], whereas in MS-based metabolomics, deconvolution reduces complexity of the data [39]. Normalization is the next step after raw data extraction. In NMR-based metabolomics, integral normalization is a standard method for normalizing and required to control possible variations in sample concentrations and variable sample dilutions [50]. Meanwhile in MS-
based metabolomics, normalization coupled with transformation is introduced to minimize the impact of variability of high-intensity peaks [39].

After normalization, the extracted data are further analyzed by multivariate data analysis. Basically multivariate data analysis can be distinguished into two general types, namely unsupervised and supervised approaches. Unsupervised approaches are often known as clustering techniques, not using independent variables, provide a simplified description of the data with describing general information, and visualize the relationship between the dependent variables [47]. Moreover unsupervised approaches are powerful methods for sample classification. Whereas supervised approaches describe the subset of variation in the dependent variables and do not describe the variation irrelevant to the experimental question, thus provide more simplified compared to unsupervised approaches [47]. Supervised approaches are appropriate for metabolomics analysis that aims to discover characteristic compounds and the sample identity is often known [39].

One of the most popular of multivariate data analysis for unsupervised approaches is principal component analysis (PCA) [55]. It is basically a data reduction technique and represents multivariate data in a low dimensional space. Furthermore PCA has capabilities for finding relationships and variances in the data, making a model of how chemical system behave, and separating an underlying systematic data from noise [56]. Figure 2 describes a graphical overview of the matrices and vectors used in PCA. In PCA, extracted data are represented by a set of new variables known as principal components (PC). Similarities and variances of samples according to metabolomic data are showed in a score plot and influence of each metabolite signal is visualized by the loading plot of PCA [17].

The other common methods have been used for unsupervised approaches are independent component analysis (ICA) and hierarchical cluster analysis (HCA). ICA apparently is an improvement of PCA, because in the beginning step it needs PCA for reduction of the high dimension of the dataset and the quality of ICA is determined by the number of principal components as well [40]. In ICA, new set of components known as independent components (ICs) are calculated to detect more meaningful components, and different ICs represent different non-overlapping information [40]. ICA has shown a good result in metabolic fingerprinting for a small number of high-dimensional samples when PCA failed to do it [40]. HCA classifies samples in a data set based on their similarity. It creates a hierarchy of clusters that is commonly visualized in a tree structure called a dendogram where the root consists of a single cluster containing all observations and the leaves correspond to individual
observations. Therefore HCA draws an easy description of the similarities of the samples within data sets [41].

Figure 2. (A) is a graphic of a data matrix X with its first two principal components. Index \( i \) is used for objects (rows) and index \( k \) for variables (columns). There are \( N \) objects and \( K \) variables. The matrix \( E \) contains the residuals, the part of data not “explained” by the PC models [56]. (B) is an example of a score plot.

Partial least squares (PLS) regression is one of the common methods for supervised approaches. PLS combines features from PCA and multiple regressions. It can be used for discrimination with creating a linear regression model by projecting the predicted and the observable variables to a new dimension. PLS has been improved to several variants that also can be used for supervised approaches, such as partial least squares discriminant analysis (PLS-DA), and orthogonal partial least squares discriminant analysis (OPLS-DA). PLS-DA can discriminate samples in a data set with identification of variables that significantly show relevant variations in the data set. OPLS-DA is an improvement of PLS-DA and removes irrelevant information and aligns the projections precisely to the aspect of interest, thus gives better interpretation than PLS-DA. This technique discriminates between two or more groups in a data set [57] in which the regression model is calculated between the multivariate data and a response variable that only contains class information [58].
Conclusions

Many factors influence the profiles of metabolites biosynthesized by medicinal plants. Different environmental conditions like soil, fertilizers, climate, pest control and insects for plant cultivation can create a high and not constant diversity of biochemical composition. However metabolomics has been proven as powerful method to discriminate and classify the same medicinal plants cultivated at different geographies, different varieties of plants, and different genotype having similar morphology by identifying the key compounds for discriminating. Moreover metabolomics has been used for monitoring elicitation process in medicinal plant cell cultures and for identifying effects of different genetic modification approaches in metabolite production of transgenic plants.

Harvesting treatment, storage conditions, extraction technique, preparation method, and packaging affect the quality of medicinal plant products significantly. Therefore, good analytical methods are required for guarantying high quality level from production to the pharmacy shelf. However metabolomics with different approaches has been successfully applied as a smart analytical method for quality control of medicinal plant products, since it offers efficient quantitative and qualitative analysis of metabolites comprehensively. On the other hand, metabolomics has been coupled with bioactivity assays to identify components responsible for bioactivity, investigate synergistic effects, and even to predict bioactivity of a medicinal plant product. Moreover metabolomics has also been applied to study efficacy of medicinal plant products with measuring urine, blood or other biofluids of addressed object (human or rats) and comparing it with the control. Furthermore the fact that organ damage could be associated with the corresponding changes in metabolite profiles leads to application of metabolomics in toxicity investigation of medicinal plant products.

All of these have shown that emergence of metabolomics has opened new opportunities to answer challenges in medicinal plant fields. Metabolomics has been proven as a breakthrough method to accelerate and streamline the analytical process of medicinal plant researches by allowing quick, efficient identification and quantification of the metabolites within the samples.
“Life is one big road with lots of signs. So when you riding through the ruts, don’t complicate your mind. Flee from hate, mischief and jealousy. Don’t bury your thoughts, put your vision to reality. Wake up and live!”

- Bob Marley