Method validation strategies involved in non-targeted metabolomics

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Abstract

Non-targeted metabolomics is the hypothesis generating, global unbiased analysis of all the small-molecule metabolites present within a biological system, under a given set of conditions. It includes several common steps such as selection of biological samples, sample pre-treatment, analytical conditions set-up, acquiring data, data analysis by chemometrics, database search and biological interpretation. Non-targeted metabolomics offers the potential for a holistic approach in the area of biomedical research in order to improve disease diagnosis and to understand its pathological mechanisms. Various analytical methods have been developed based on nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) coupled with different separation techniques. The key points in any analytical method development are the validation of every step to get a reliable and reproducible result and non-targeted metabolomics is not beyond this criteria, although analytical challenges are completely new and different to target methods. This review paper will describe the available validation strategies that are being used and as well will recommend some steps to consider during a non-targeted metabolomics analytical method development.

1. Introduction

Metabolomics was defined by Oliver Fiehn as the global unbiased analysis of the small-molecule metabolites present within a biological system in an identified and quantified manner [1]. In parallel, Jeremy Nicholson coined the term metabolomics, which is defined as the comprehensive and simultaneous profiling of metabolites and their effective changes resulting from different conditions such as diet, life style, genetic or environmental factors [2]. Currently both terms are used interchangeably. This methodology offers the potential for a holistic approach to clinical medicine, as well as improving disease diagnosis and understanding of pathological mechanisms. The exact definition of the metabolome is in some debate, however in general can be thought of as the complete compliment of all the low-molecular weight molecules (<1500 amu) present in the biological compartment in a particular physiological state under a given set of environmental conditions [3]. Metabolomics may also be the methodology for biomarker discovery. Biomarker was defined in 1998 by the National Institutes of Health Biomarker Definitions Working group as: “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” [4]. To make this definition more clear in the year of 2010 the World Health Organization suggested that a biomarker is “almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical or biological. The response may be functional and physiological, biochemical at the cellular level, or a molecular interaction” [5]. A biomarker could be anything such as, physical traits (body temperature, blood pressure, etc.) or presence of biological molecules in tissues or body fluids. The fundamental goal of biomarker identification in biomedical research is the discovery of a molecular signature which can correlate with a specific disease type that can be used as early diagnostic tools in clinical practice [6]. This type of marker requires high sensitivity and specificity. The metabolomics field has a key role in screening chemical markers and this approach can mainly be divided in two categories, targeted and non-targeted. Targeted metabolomics consists of the quantification of one or a set of known metabolites, which are generally related to a specific pathway or biological activity [1]. It enables exact quantification of the metabolite by employing authentic analytical standards and only focus on the changes of the quantitated metabolites [7,8]. On the other hand, non-targeted approach is the data driven, rapid high-throughput analysis of all possible metabolites present in a given set of samples without any prior knowledge of the metabolites [9]. Compared to targeted approach, in non-targeted metabolomics, it is not
possible to quantify due to the larger number of variables and because the identity of the metabolites is often unknown [10–12]. The key advantage of global approach over targeted approach is, that it enables novel areas of metabolism to be identified [8,13]. Numerous analytical platforms have been used in non-targeted metabolomics applications, such as nuclear magnetic resonance spectroscopy (NMR), Fourier transform–infrared spectroscopy and mass spectrometry (MS) coupled to separation techniques, or using direct flow injection [2,14,15]. The potentiality of NMR in high throughput metabolomics, is the minimal sample pre-treatment requirement, obtaining highly quantitative and reproducible data, however with this technique only medium to high abundance metabolites can be detected and challenges still remain for complex mixtures. MS-based metabolomics offers analyses with high selectivity and sensitivity, moreover positive and negative ionization will increase the variety of metabolites, either using direct infusion or in combination with separation techniques choosing from liquid chromatography (LC), gas chromatography (GC) and capillary electrophoresis (CE). Use of separation techniques also reduces the complexity of the mass spectra and delivers additional information on the physico-chemical properties of the metabolites [16]. However, MS-based techniques usually require sample preparation steps and analytical method development, which should be valid enough to get effective results. LC–MS based analysis is the most widely used analytical platform in non-targeted metabolomics due to its high sensitivity and selectivity. Separation with LC can reduce ion suppression caused by co-eluting compounds, isobaric interferences and often can separate isomers. In addition, analytical separation with LC could be benefited from lower detection limits and improved MS data quality due to reduced background noise. The combination of GC with MS provides high-resolution, analyte-specific detection, and quantification of metabolites and as well it has the capability to identify unknowns. However, a major prerequisite for GC–MS analysis is a sufficient vapor pressure and the analytes should be thermally stable. The sample pre-treatment steps for GC–MS analysis are quite long and only volatile metabolites can be analyzed, which are the main limiting factors for this technique. CE is a powerful technique for the separation of charged metabolites, offering high-analyte resolution. The combination of CE with MS makes CE–MS an ideal tool for the analysis of polar compounds present in the metabolome. However, only a few applications have been published as it is not a generally available technique in the laboratory.

Accordingly, a single analytical approach is not enough to cover the entire metabolome. Thus the integration of multiplatform approaches are necessary to circumvent this issue and MS detection coupled to separation techniques along with NMR are becoming the most relevant tools in this aspect. Multiplatform metabolomics has been applied on different biological specimens either for targeted or non-targeted analysis. The multiplatform approach was well demonstrated in a study by Psychogios et al., while establishing human serum metabolome [17]. In order to achieve coverage of 4229 metabolites, six distinct analytical platforms were used, including high-resolution NMR, GC–MS, LC–MS (positive and negative ionization) and direct flow injection in MS.

After instrumental considerations another critical step in metabolomics approach is the biostatistics (univariate and multivariate data analysis) which reflects the differences between metabolomes and characterize specific phenotypic characteristics. However the correct biological interpretation of a specific metabolite difference in non-targeted approach depends on the reliability and suitability of the entire approach (from sample treatment to biomarker identification). Hence, the term validation comes in concern. The objective of any analytical measurement is to obtain consistent and reliable data avoiding false positive and negative discoveries. Validated analytical methods play a major role in achieving this goal. Results from method validation provide the picture of the quality, reliability and consistency of analytical results, which are fundamental for any good analytical practice. Method validation has received considerable attention since many years in the literature. For targeted metabolomics, there are several validation guidelines available, especially focused on instrumental aspects, while for non-targeted approach there are no guidelines available and they will only be promoted by the research community, because regulatory agencies are mainly focused on manufactured products. However, researchers are using several alternative ways to validate non-targeted approaches. The aim of this review paper is to discuss about the different validation criteria that are being used in analytical methods for non-targeted metabolomics and as well proposing validation steps to carry out this type of analysis.

2. Concept of validation

In the mid of 1970s in order to improve the quality of pharmaceuticals, the concept of validation was first proposed by the Food and Drug Administration (FDA) [18]. According to FDA validation is “Establishing documented evidence that provides a high degree of assurance that a specific process will consistently produce a product meeting its pre-determined specifications and quality attributes” [19]. A properly designed system will provide a high degree of assurance that every step, process and change has been properly evaluated before its implementation. Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. It is the process of defining an analytical requirement and confirms that the method under consideration has performance capabilities consistent with what the application requires. Results from method validation can give an overview about the method quality. Quite often method validation evolves from method development and so the two activities are closely tied.

Method validation has received considerable attention in the literature and there are several guidelines available for analytical and bio-analytical aspect and they are as follows:

a. The United States FDA established two industrial guidelines. First one for the validation of analytical methods (this guidance provides recommendations to applicants on submitting analytical procedures, validation, data and samples to support the documentation of the identity, strength, purity and potency of drug substances and drug products) and second one for the validation of bioanalytical methods (this guidance applies to bioanalytical methods used for human or non-human clinical, pharmacological, toxicological studies and preclinical studies-based on bioanalytical procedures such as chromatography, immunology and microbiology) [20,21].

b. ICH developed two guidelines for method validation that were later merged in one: Q2-R1. It discusses the considered characteristics (terminology and definitions) and methodology to be used during the validation of the analytical procedures [22].

c. International Union of Pure and Applied Chemistry published “Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis”. This guideline provides minimum recommendations on procedures that should be employed to ensure adequate validation of analytical methods [23].

The above-described guidelines are mainly focused on seven common parameters that should be considered during bioanalytical method validation in order to establish the method “fit-for-purpose”. The definition for these seven parameters has been included in Table 1.
3. Non-targeted metabolomics and method validation

Metabolomics is growing rapidly with the major advances not only in analytical tools (mainly NMR and MS) but also in chemometrics. The goal of non-targeted approach is to determine all possible metabolites differentially expressed under the conditions that are compared in a study, with the potential to detect and identify candidate chemical biomarkers involved in a specific condition. The steps involved in non-targeted metabolomics includes: (i) selection of biological sample, (ii) sample pre-treatment, (iii) instrumental analysis of prepared samples using NMR or MS coupled with LC, GC or CE, (iv) data treatment and statistics (univariate and multivariate), and (v) identifying the important discriminating metabolites using commercial database or analytical tools (Fig. 1).

Non-invasive/minimally invasive sample types, principally urine and serum/plasma are often used in metabolomics, as these are the best accessible and it is supposed that the end product of any metabolic changes are seen in biological fluids. Non-targeted metabolomics has been successfully applied on serum/plasma and the authors were able not only to distinguish case and control but also to establish strong biomarkers in many diseases [24,25]. Using non-targeted approach several studies have found urinary markers, in subjects either treated with a drug or a specific dietary consumption or microorganism contaminated or in specific diseased concern [26–28]. Beside urinary and plasma metabolomics, tissue analysis is also important because not all changes affect in the same direction at tissue level or vice versa which has been seen in several studies and that is important mainly for mechanism research and in animal models [29–33]. Metabolic changes in specific disease state are first seen at the tissue site. Moreover, pairwise comparison of diseased and control tissue regions could provide strong markers. Hence, tissue metabolomics can take a big part in non-targeted research [34–36]. Non-targeted approach has also been applied on different other sample type such as saliva, bile extract, cell lines, microbial extract, food extract or even in environmental toxicology [37–43]. To understand a disease mechanism perfectly researchers have analyzed not only a single biological fluid but also a combination of urine, plasma and tissue to see the proper molecular mechanism [31,32,44–46].

An important number of papers have also been published on plant applying non-targeted approaches, including flower plant, herbal medicine, diseased plant, food industry, etc. [47–49], however analytical aspects are quite different and they are out of the focus of this review.

In addition to the selection of the proper type of sample or combination of them, another question appears, as no single analytical technique is suitable for the detection, identification and quantification of all the metabolites with varying physicochemical properties in non-targeted analyses. Thus multiphase approach has also been applied choosing from NMR, LC, GC or CE and combination of LC × LC and GC × GC are also being used [36,50–55]. Sample pre-treatment is a pre-requisite for any analytical technique. Thus improved sample preparation techniques are as well in concern to get wider metabolite coverage along with easy preparation steps and minimal sample volume [56–58]. After sample treatment and instrumental consideration, data treatment comes in the next row and many research lines try to contribute to establishing or upgrading the data processing algorithms in order to get actual numbers [59–62].

The term validation describes the meaning by itself: “valid for a purpose”. Therefore a valid method needs first and for all a clear description of the purpose of which it is intended to be used. There are no guidelines for validating analytical part in non-targeted approach. The purpose of non-targeted metabolomics is to find statistically significant biomarkers through unbiased differential analysis of as many signals as possible coming from biological samples which will compare different situations being studied. That purpose will determine the validation parameters considering the facts that the accurate quantification is not an aim and standards are not included in the analysis. Comparisons will be valid as long as all the samples are studied under the same conditions under a precise method and the change in the signal should be related to the concentration of the components.

Prior to method validation, the key point is to develop a method including all necessary steps and with special attention on sample pre-treatment, that should be the minimum possible to make it compatible with the instrumental technique. Highly precise, broad metabolite coverage will prove the applicability of the method for non-targeted approach [36].

Subsequently, researchers are using some alternative approaches to validate the analytical methodology. The recent validation strategies are described as follows.

### 3.1. Quality control samples (QCs)

When using high-throughput techniques for non-targeted approach, some potential limitations arise which should be kept in mind while developing and applying such technology. Such limitations are the potential for drift in both chromatographic and MS performance, the repeatability and reproducibility of the method. These drawbacks should be recognized and controlled in order to eliminate bias due to a gradual change in the performance of the system. To monitor the performance of the instruments researchers have described to randomize the order of the samples and include

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<td><strong>Criteria</strong></td>
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quality control samples (QCs) [63–65]. QCs are a pool of sample made from the biological test samples to be studied, or a representative bulk control sample which should be assessed against predefined criteria to enable acceptance or rejection of the analytical run. It is also strongly advisable to implement a number of injections at the beginning of the analytical run to stabilize the system and at the end and as well they should be repeatedly analyzed at regular intervals throughout the sequence run. This would subsequently explain whether the system is or not stable along the entire sequence run and will describe the quality and integrity of the analyzed data sets. FDA has proposed other useful criteria for bioanalytical methods validation to calculate the relative standard deviation (RSD) of the molecular features or possible compounds present in QCs [66]. For non-targeted metabolomics analysis by LC–MS values close to 30% RSD could be allowed (clearly highly variable ions, with RSD higher than 30%, would not be good candidates as biomarkers). Along with checking RSD in QCs, researchers are trying to validate the method by checking the linearity of diluted pooled QCs. Matrix effects and recovery were checked as well in the QCs [67,68]. Checking the linearity in diluted QCs helps to get clean data set based on the criteria that only those metabolites which pass a linear trend will go for further data analyses. However, there are some metabolites whose concentrations are very low and will not show a linear trend or sometimes too much diluted that will be under LOD. This can lead us to lose these metabolites. In addition, the dilution of the matrix makes that linearity not fully reliable, because ion suppression will be different to real samples. In any case, the use of QCs in non-targeted metabolomics analysis can be considered as equivalent to the use of standards in routine target analysis, more than validating a method. In a review by Dunn et al. the importance of QC samples in non-targeted MS based metabolomics has been well explained [69].

3.2. Spiked standards

Using the external standard method is very common in pharmaceutical industry in order to check the reliability of the methodology. Several researchers have followed this validation criterion in non-targeted analysis. Pereira et al. used this criterion somehow common with the quantitative method validation in nutritional plasma metabolomics by calculating the matrix effect and recovery after spiking six different xenobiotic standards [70]. However, for large batch analysis the method was validated in terms of the reproducibility of retention times and accurate masses in QCs. In another strategy, analysis of the analytical reproducibility within a run was performed by monitoring sample specific identified metabolites with different physico-chemical properties including known endogenous metabolites along with xenobiotics [71]. Nonetheless it needs to be stated here that although spiking some selected metabolites could give a good approach to the general behaviour of the method, however results cannot be assumed for all the components in the sample. The use of internal standards has also been applied to check instrumental reproducibility [36,72].

Conventional validation guidelines were as well followed in several strategies. Non-targeted metabolomics provides information of huge number of metabolites present in a specific sample type. It is not possible to use all of them to validate the method. However it would be good to target some of them from the non-targeted metabolites (considering different class/category, retention time/migration time, more abundant or less abundant, various physico-chemical properties) and validate the methodology. Though this validated method is not enough for validating a final biomarker for clinical use, which requires a target method with a proper, classical method validation, but results are more reliable compared to non-validated methods at least for further data processing. There are very few papers that used these criteria not only to check method reproducibility but to successfully validated and applied on bio-fluids or tissues [36,72–76].

3.3. Statistical model validation

In disease screening, non-targeted metabolomics results are ended-up with a prediction of class belonging, using multivariate statistical algorithm, usually first applying non-supervised principal component analysis (PCA). PCA provides summery of all the variables entering in the statistical analysis by finding correlation among the variables. Following correlation, it reduces the variables into a smaller number of principal components which is responsible for the possible variance in the observed variables. Finally PCA expressed the whole data sets in a global and qualitative visual pattern, highlighting similarities and differences between and within the sample [77,78]. In metabolomics, either targeted or non-targeted, the quality of the instrumental analysis is usually checked by the tight clustering of QCs in the PCA model. By the poor or good clustering of QCs, a preliminary idea of the data could be obtained. Before making a PCA model the QCs can be excluded and later they are predicted on the same model. The clustering of the QCs after prediction shows the repeatability of the instrumental performances as well it describes that the separations between the groups are not due to the instrumental variations but due to the sample itself [64]. However, an in depth discussion of statistical model validation is out of the scope of this paper as this review article is devoted to non-targeted metabolomics analytical method validation.

3.4. Other parameters

In addition to the criteria described above some researchers are using alternative ways to qualify the analytical method by examining the system performance checking the signal to noise ratio, plate number, retention factor, etc. either in pooled samples or by diluting the pooled samples [67,68,79]. Whereas, using these strategies only relative quantification of metabolites are possible.
4. Recommended validation criteria for non-targeted approach

The goal of non-targeted metabolomics (which utilizes all the possible metabolites that are changing in specific conditions) includes the analysis of biological samples in an analytical platform and the generation of a predictive model from a collection of detected multiple compounds, which used to classify new samples/persons into specific groups (e.g., control vs. case) with optimal sensitivity and specificity. Finally, the significant metabolites should be related to biological pathways in order to identify potential markers and their biological meaning. Thus a thoroughly controlled, validated analytical method should consider while developing a non-targeted approach and the validation should be based on the primary objective of the research. In Fig. 1, the general steps involved in non-targeted approach, the red circle with marked a, b and c steps should be monitored and validated in order to get a reproducible and sensitive result. In this review paper we are only focusing on the validation steps involved in stage “a”: From sample selection to analytical method development.

(a) From sample selection to analytical method development:

(i) Selection of sample: In any research study including non-targeted metabolomics to define the biological variation accurately and validly a set of study population is the first requirement, whether it is small or large needs to be monitored carefully. For non-targeted approach, the first step is to select samples minimizing the noncontrolled variations among them as much as possible (such as age, sex, body mass index and any medical conditions), then randomly select a minimally sufficient set of samples. It is necessary to check that groups have equal scores on the dependent variable at the start of the study and are randomly assigned, extraneous variables are controlled so no group is affected by them during the analysis and each group receiving identical treatment.

(ii) Sample treatment and analytical conditions: The need of method development arises the necessity of method validation and proper sample treatment. One of the critical steps in non-targeted approach is the sample preparation with important consequences on the accuracy of the result. The choice of sample treatment depends on the analytical tools and the metabolites of interest. For non-targeted metabolomics, an easy sample pre-treatment should be focused to get broader analyte signals compatible along the techniques with appropriate sensitivity and reproducibility in terms of retention/migration time, abundance, pressure (LC) and current (CE) and minimizing carry over effects (LC). However, for biofluids the sample preparation is relatively easy where as for cell or tissues or extracts, special attention are needed to cover wider metabolites. For small scale study the sample preparation can be done on the day of analysis, however this is not possible for large scale study. The sample preparation for large scale can be done in two ways – preparing all of them in one day and analysing in different days or dividing them in several batches and prepare every day before analysis. The design of the sample pre-treatment will completely be dependent on the objective of the study. Despite this, careful attention should be taken during the set up of analytical conditions. Consideration should be focused to get reliable and reproducible data acquisition along with broader signal range [80]. Several protocols are available on urine, plasma/serum and tissue metabolomics analysis [81–83].

(iii) Analytical method validation: Non-targeted metabolomics helps to see the changes in all the possible metabolites identified and to interpret for the specific conditions. So a reliable analytical method could give us a primary reliable assumption from the identified distinctive metabolites. The fundamental of any analytical technique is to obtain dependable, consistent and accurate data. As previously described in Section 3, an analytical non-targeted method can be validated by following steps:

First, following the published guidelines for targeted metabolomics, some known compounds that have already been identified in the specific sample should be selected (considering different physic-chemical properties, intensities along the entire chromatogram). If the method is validated in terms of linearity, accuracy and precision for the selected compounds, it can be considered that the non-targeted approach will generate reliable and acceptable data sets for further evaluation. Moreover, sample treatment and method development steps should be optimized.

For routine work it is recommended the use of QCs, which are theoretically identical biological samples, with a metabolic and sample matrix composition similar to those of the biological samples under study. Two types of QCs are being used in metabolomics: (i) pooled QCs where small aliquots (same volume) of each biological sample to be studied are aliquoted and thoroughly mixed and (ii) commercially available biological samples. Pooled QCs are useful as they are very close to the composition of the biological sample to be studied. The signal variations of any metabolite could be reflected in the QCs. However, pooled QCs can only be applicable in small scale studies whereas for large batch analysis it is not possible to aliquot in order to make a pool for QCs. Therefore, a replacement for QCs is required. As the replacement QCs does not completely resemble the original population, there are some metabolites which will not be seen in there. During data processing RSDs for these metabolites will be unknown and they could be removed during data filtering, leading to a loss of metabolites which could be important biomarkers. QCs help to measure the repeatability within an analytical run and allow filtering data before analysis considering the drift in signal variations, retention time and accurate mass. QCs are being used in metabolomics for three reasons: The first is to ‘condition’ or equilibrate the analytical system with injections of matrix prior the main analytical run, to ensure that reproducible data are acquired. Secondly, it provides data to calculate analytical and technical precision. And thirdly the data from QCs are used for signal corrections within or between analytical run/batch analyses. An analytical method can be validated by diluting the pooled QCs and checking the linearity. By doing so, the metabolites which are showing a linear trend can be considered for further data processing. There are some limitations in this methodology that have been previously commented.

To get a snapshot about the instrumental repeatability before starting any data treatment the total signal from each chromatogram could be plotted against each sample (as per the sequence/run order) as shown in Fig. 2. The dispersion of QCs in the plot could reflect the instrumental repeatability.

Moreover, for validation it is recommended to use blanks during method development. Blanks should contain the solvent resembling the study samples and will be processed following the same procedure along with the real samples. The goal of including blanks is to detect and minimize the carryover during method development and consider the identified features in blanks as background, mainly for GC–MS.

Another indirect way of analytical method validation is to check the signal variation in all identified metabolites in terms of relative standard deviations in QCs and as well filtering data
5. Conclusion

Non-targeted metabolomics may advance our discovery phase in diagnosis, treatment and prognosis of diseases. Using this approach, disease-related mechanisms may be uncovered, independent of the pathways involved and chemical diagnostic biomarkers in biological samples can be characterized. The analytical methods applied in metabolomics latest approaches need to be qualified in terms of validation. Thus there is a need for a well-defined validation criterion to perform a non-targeted study controlling false discoveries. Different efforts have been made by some research groups in order to define the validation parameters with some accepted ranges after following established methodologies, though up to now validation strategies for non-targeted approach are not well defined. Once the method has been optimized for the broadest physiochemically different types of compounds, the clues for validation of the analytical method in non-targeted metabolomics are obtaining objective evidences about the number of possible compounds following defined criteria of precision and linearity by spiking or by dilution of the QC. Metabolomics method validation should now be considered as an open discussion among the researchers.

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