3. Dietary exposure biomarkers in nutritional intervention and observational studies to discover biomarkers of intake and disease risk through an HPLC-QToF-MS metabolomics approach

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Abstract. Health is highly influenced by food intake. Nutrimetabolomics has been proposed as a tool for assessing the changes in metabolome associated with food consumption and/or the effects of a dietary intervention. In this chapter, we have summarized the most relevant results of our recent research on the identification of biomarkers related to food ingestion (biomarkers of intake), as well as their potential association with health (biomarkers of effect), through the application of an untargeted HPLC-QToF-MS metabolomics approach in nutritional studies.
with different designs. The results have shown that diet-related differences in urinary metabolome are associated with food digestion, microbiota metabolism and endogenous metabolism; and the predictive capacity of dietary exposition can be improved using multimetabolite combined models compared with the use of single compounds.

**Introduction**

Evaluation of the effects of food on health requires results to be obtained in studies that allow conclusions to be reached with the maximum degree of scientific evidence and, based on this information, solid and reliable recommendations to be elaborated for consumers. For this reason, precise measurement of dietary intake is a crucial factor in studies that analyse the relationships between diet and health. Traditionally, dietary intake data have been obtained from food surveys. The most commonly used methods are food frequency questionnaires, 24-hour recalls and dietary records. However, in spite of being the most frequently used methods, they present a series of methodological limitations due to systematic and random errors [1, 2]. These drawbacks may attenuate the relative risk estimates and decrease the statistical power of the studies [3, 4] and it has been pointed out that they are one of the causes of some of the reported inconsistencies between food and health in the scientific literature, since the effects of diet on risk factors may be distorted due to errors in the assessment of intake (in the case of observational studies) or due to a lack of compliance with the assigned nutritional intervention (in the case of intervention studies) [5].

Faced with this situation, and given the need to obtain a more precise intake assessment, nutritional biomarkers have emerged as a precise and objective tool for the determination of dietary exposure that could complement the data obtained from food surveys [6]. Biomarkers have been defined 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” [7]. Biomarkers can be divided into three categories: i) exposure biomarkers, defined as those exogenous compounds, or some of their metabolites, that can be measured in a biological sample of the organism; ii) effect biomarkers, defined as those measurable elements related to a biochemical or physiological alteration in the organism that, depending on the magnitude, may be associated with a possible deterioration in health or disease; and (iii) susceptibility biomarkers, defined as those substances that indicate the body’s ability to respond to a particular exposure [8, 9].

Within the field of food sciences, a nutritional biomarker is any biochemical, functional or clinical indicator measured in a biological sample
that reflects the nutritional status with respect to the intake or metabolism of dietary components, as well as the biological consequences of food intake [10]. An ideal dietary biomarker should accurately indicate the level of intake and should be specific, sensitive and applicable to a large number of populations [6]. In this respect, the most important criteria to take into account when using dietary biomarkers are summarized in Table 1.


<table>
<thead>
<tr>
<th>Biological considerations</th>
<th>Sample</th>
<th>Methodology</th>
<th>Relation to the exposure</th>
<th>Sensitivity and specificity</th>
<th>Dose-response</th>
<th>Time of exposure</th>
<th>Population</th>
<th>Interindividual variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analytical methods</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>It is necessary to define the type of sample, the time of its collection, and the conditions of storage and preparation.</td>
<td>The marker should be quantifiable by a defined method and the analytical error should be known.</td>
<td>The association between food intake and the exposure marker should be causal. That is, the marker should be a known compound (or a metabolite) present in the food.</td>
<td>The biomarker should be as particular of the evaluated food as possible, so that the percentage of true positive values for exposure (sensitivity), and true negative values for non-exposure (specificity), should be as high as possible.</td>
<td>There should be a positive association between the level of exposure and the measured level of the biomarker.</td>
<td>It should be determined whether it is a short-term (reflects recent consumption of food) or long-term (reflects habitual intake) exposure marker.</td>
<td>The population in which the exposure marker can be applied should be known.</td>
<td>The main potential sources of interindividual variation (such as genotypes, gender, age, smoking, microbiota, etc.) should be investigated.</td>
</tr>
</tbody>
</table>
In the investigation of new biological markers related to diet, metabolomics has emerged as a powerful tool for the discovery of new nutritional biomarkers of intake and effect [14]. Metabolomics is the science that studies the metabolome, i.e. the set of metabolites (defined as those intermediate molecules and products of metabolism with a molecular weight less than 1500 Da) present in a biological system (cell, tissue or fluid) [15–17]. The diet influences two fractions of the human metabolome: i) the food metabolome, which includes all external metabolites derived from dietary exposure; and (ii) the endogenous metabolome, which includes all the metabolites produced by the organism [18]. In recent years, there has been a significant increase in the number of publications in this field (Fig. 1). This demonstrates the growing interest that is being devoted to this discipline, as well as the potential it offers in research.

With the introduction of metabolomics in the field of nutritional research, the concept of nutritional metabolomics, or nutrimetabolomics, has emerged. It has been defined as the omics discipline that studies how the diet affects the whole metabolome [19].

Feeding induces changes in the metabolism of the organism, which can be evaluated by the analysis of the endogenous and exogenous metabolites in biofluids. These metabolites can be used as objective and accurate biomarkers of food consumption and/or the effects of a dietary intervention. The food metabolome includes all metabolites derived from food intake, their

![Figure 1](image_url). Number of publications per year appearing in PubMed using the search “metabolomics” (the number of publications resulting from the search “metabolomics & nutrition” in the same database is indicated in orange).
absorption and their biotransformation in the tissues or organs or by the microbiota [20]. In light of this, the application of metabolomics in nutritional studies has become a new strategy in obtaining new biomarkers related to the evaluation of the nutritional status of an individual, food consumption, biological consequences produced after a nutritional intervention, or the study of metabolic mechanisms in response to diet according to a specific metabolic phenotype [13, 17, 21]. The food metabolome has a high complexity and variability, since it is estimated that food contains > 25,000 different compounds, the great majority of which will undergo various metabolic processes in the organism [18]. This particular characteristic makes the food metabolome a very important source of information about the diet of individuals, and its characterization would enable eating habits to be monitored in an objective and precise way, and the influence food has on the risk of developing diseases could be studied [18].

Herein we will summarize the main results of our recent research on the discovery of new biomarkers of dietary exposure in a population from the Mediterranean region at high risk of cardiovascular disease through the application of an untargeted HPLC-QToF-MS metabolomics approach in nutritional studies with different designs. Different foods habitually consumed within the dietary pattern of the studied populations were selected. Specifically, the metabolic fingerprint of white and whole-grain bread [22], nuts [23, 24] and cocoa [25, 26] was analysed.

1. HPLC-QToF-MS untargeted metabolomics analyses

1.1. Identification of biomarkers of bread consumption in a free-living population

The objective of this work was to identify biomarkers of bread consumption by applying a nutrimetabolomics strategy [22]. Using an untargeted HPLC-QToF-MS approach together with multivariate analysis, the urine of 155 free-living individuals stratified into three groups according to their usual bread consumption was analysed (56 non-bread consumers, 48 consumers of white bread and 51 consumers of whole-grain bread).

The most differentiated metabolites (VIP ≥ 1.5; Table 2) included plant phytochemical compounds, such as benzoazinoid and alkylresorcinol metabolites, and compounds produced by gut microbiota (such as enterolactones, hydroxybenzoic and dihydroferulic acid metabolites). Pyrraline, 3-indolecarboxylic acid glucuronide, riboflavin, 2,8-dihydroxyquinoline glucuronide and N-α-acetylcitruline were also tentatively identified.
Table 2. Metabolites identified with different bread consumption levels.

<table>
<thead>
<tr>
<th>RT (min)</th>
<th>Detected mass (m/z)</th>
<th>Assignation</th>
<th>Identification</th>
<th>NBC vs WHB</th>
<th>NBC vs WGB</th>
<th>WHB vs WGB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NCB</td>
<td>NBC</td>
<td>WHB</td>
</tr>
<tr>
<td>0.88</td>
<td>188.0049</td>
<td>[M–H]-</td>
<td>2-Aminophenol sulphate</td>
<td>↑ ↑ –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>1.48</td>
<td>328.1036</td>
<td>[M+H]+</td>
<td>HPAA glucuronide</td>
<td>↑ ↑ –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td></td>
<td>326.0651</td>
<td>[M–H]-</td>
<td>–</td>
<td>↑ ↑ –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>2.07</td>
<td>168.0609</td>
<td>[M+H]+</td>
<td>HHPAA</td>
<td>– ↑ ↑</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>3.40</td>
<td>372.0925</td>
<td>[M+H]+</td>
<td>HMBOA glucuronide</td>
<td>↑ – –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td></td>
<td>370.0772</td>
<td>[M–H]-</td>
<td>↑ ↑ –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>3.68</td>
<td>326.0922</td>
<td>[M–H]-</td>
<td>HBOA glycoside</td>
<td>– ↑ ↑</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>3.72</td>
<td>152.0671</td>
<td>[M+H]+</td>
<td>HPPA</td>
<td>– ↑ –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>4.78</td>
<td>196.0596</td>
<td>[M+H]+</td>
<td>HMBOA</td>
<td>↑ ↑ –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td></td>
<td>194.0410</td>
<td>[M–H]-</td>
<td>↑ ↑ –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
</tbody>
</table>

**Benzoxazinoid-related compounds**

<table>
<thead>
<tr>
<th>RT (min)</th>
<th>Detected mass (m/z)</th>
<th>Assignation</th>
<th>Identification</th>
<th>NBC vs WHB</th>
<th>NBC vs WGB</th>
<th>WHB vs WGB</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.85</td>
<td>357.0791</td>
<td>[M–H]-</td>
<td>DHPPA glucuronide</td>
<td>↑ ↑ ↑</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>3.12</td>
<td>233.0118</td>
<td>[M–H]-</td>
<td>3,5-Dihydroxyphenylethanol sulphate</td>
<td>– ↑ –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>5.75</td>
<td>289.0412</td>
<td>[M–H]-</td>
<td>DHPPTA sulphate</td>
<td>– ↑ ↑</td>
<td>– – –</td>
<td>– – –</td>
</tr>
</tbody>
</table>

**Alkylresorcinol metabolites**

<table>
<thead>
<tr>
<th>RT (min)</th>
<th>Detected mass (m/z)</th>
<th>Assignation</th>
<th>Identification</th>
<th>NBC vs WHB</th>
<th>NBC vs WGB</th>
<th>WHB vs WGB</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.67</td>
<td>313.0588</td>
<td>[M–H]-</td>
<td>Hydroxybenzoic acid GlcA</td>
<td>↑ ↑ –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>4.72</td>
<td>275.0219</td>
<td>[M–H]-</td>
<td>Dihydroferulic acid sulphate</td>
<td>– ↑ ↑</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>6.32</td>
<td>299.1278</td>
<td>[M+H–GlcA]+</td>
<td>Enterolactone glucuronide</td>
<td>– ↑ ↑</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td></td>
<td>473.1447</td>
<td>[M–H]-</td>
<td>– ↑ ↑</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
</tbody>
</table>

**Microbial-derived metabolites**

<table>
<thead>
<tr>
<th>RT (min)</th>
<th>Detected mass (m/z)</th>
<th>Assignation</th>
<th>Identification</th>
<th>NBC vs WHB</th>
<th>NBC vs WGB</th>
<th>WHB vs WGB</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.73</td>
<td>255.1345</td>
<td>[M+H]+</td>
<td>Pyrraline</td>
<td>– ↑ –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td></td>
<td>253.1172</td>
<td>[M–H]-</td>
<td>– ↑ ↑</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
</tbody>
</table>

**Markers of heat-treated food products**

<table>
<thead>
<tr>
<th>RT (min)</th>
<th>Detected mass (m/z)</th>
<th>Assignation</th>
<th>Identification</th>
<th>NBC vs WHB</th>
<th>NBC vs WGB</th>
<th>WHB vs WGB</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.25</td>
<td>338.0871</td>
<td>[M+H]+</td>
<td>3-Indolecarboxylic acid</td>
<td>– ↑ ↑</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td></td>
<td>336.0697</td>
<td>[M–H]-</td>
<td>glucuronide</td>
<td>– ↑ ↑</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>4.65</td>
<td>377.1475</td>
<td>[M+H]+</td>
<td>Riboflavine</td>
<td>↑ ↑ ↑</td>
<td>– – –</td>
<td>– – –</td>
</tr>
</tbody>
</table>

**Other exogenous metabolites**

<table>
<thead>
<tr>
<th>RT (min)</th>
<th>Detected mass (m/z)</th>
<th>Assignation</th>
<th>Identification</th>
<th>NBC vs WHB</th>
<th>NBC vs WGB</th>
<th>WHB vs WGB</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.63</td>
<td>218.1140</td>
<td>[M+H]+</td>
<td>N-α-Acetylcitrulline</td>
<td>– ↓ –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>4.20</td>
<td>338.0882</td>
<td>[M+H]+</td>
<td>2,8-Dihydroxyquinoline</td>
<td>– ↑ ↑</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td></td>
<td>160.0382</td>
<td>[M–H–GlcA]-</td>
<td>glucuronide</td>
<td>– ↑ ↑</td>
<td>– – –</td>
<td>– – –</td>
</tr>
</tbody>
</table>

DHPPA, 3-(3,5-dihydroxyphenyl) propanoic acid; DHPPTA, 5-(3,5-dihydroxyphenyl) pentanoic acid; GlcA, glucuronide; HBOA, 2-hydroxy-1,4-benzoxazin-3-one; HHPAA, 2-hydroxy-N-(2-hydroxyphenyl) acetamide; HMBOA, 2-hydroxy-7-methoxy-2H-1,4-benzoxazin-3-one; HPAA, N-(2-hydroxyphenyl) acetamide; HPPA, 2-hydroxy-N-(2-hydroxyphenyl) acetamide; NCB, non-consumers of bread; RT, retention time; WGB, whole-grain bread consumers; WHB, white-bread consumers.

↑ indicates significantly higher levels in the second group of the comparison; ↓ indicates significantly lower levels in the second group of the comparison.
A stepwise logistic regression analysis was used to combine several metabolites in a multimetabolite model to predict bread consumption. ROC curves were constructed to assess the predictive capacity of both the individual metabolites and their combination (multimetabolite models). The values of the area under the curve [AUC (95% CI)] of the combined models ranged from 77.8% (69.1% – 86.4%) to 93.7% (89.4% – 98.1%), whereas the AUCs for the metabolites included in the prediction models had weaker values when they were evaluated individually. The AUCs ranged from 58.1% (46.6% – 69.7%) to 78.4% (69.8% – 87.1%).

The results of this study demonstrated that a daily bread intake has a significant impact on the urinary metabolome, although this is evaluated in free-living conditions. It was also shown that the predictive ability of a combination of various biomarkers of dietary exposure is better than using single biomarkers.

1.2. Nutritional biomarkers of regular nut consumption in intervention and observational studies

Healthy effects of nuts have been attributed to their particular chemical composition. Monitoring metabolites present in biological samples after nut consumption could help to unveil the pathways involved in the effects of this food on the human organism. Therefore, changes in the urinary metabolome of patients with metabolic syndrome undergoing a 12-week nutritional intervention with a daily intake of 30 grams of nuts were determined through an untargeted metabolomics approach [23]. In line with this study, the urinary metabolome of habitual consumers of walnuts in free-living conditions was characterized using the same methodology [24].

This strategy revealed several markers associated with nut intake in both studies. They included markers of fatty acid metabolism, phase II and microbial-derived metabolites of nut polyphenols, and intermediate metabolites of the tryptophan/serotonin metabolic pathway. The increased excretion of serotonin metabolites was associated with nut consumption for the first time in the intervention study [23] and some of them were replicated in the observational study [24].

In the observational study, subjects were divided into two groups (training and validation sets) and a stepwise logistic regression analysis was used to select a multimetabolite prediction model for walnut exposure in the training set [24]. The predictive model of exposure to walnuts included at least one component of each class. The AUC (95% CI) for the combined biomarker model was 93.5% (90.1% – 96.8%) in the training set and 90.2% (85.9% – 94.6%) in the validation set. In contrast, the AUC values for individual metabolites were ≤ 85% in all cases (Fig. 2).
1.3. Analysis of the metabolic footprint of cocoa product exposure in studies with different designs

An interventional and an observational study were developed for the study of biomarkers of habitual consumption of cocoa. The design of intervention study was a randomized, crossover and controlled 4-week clinical trial involving 22 participants [27], whereas in the observational, the urinary metabolome of 32 consumers of cocoa products and 32 matched subjects not consuming cocoa was profiled [28]. In the nutritional intervention study, subjects received 40 g/day of cocoa powder in 500 mL of skimmed milk or 500 mL/day of skimmed milk as control. Twenty-four-hour urine samples were collected at the beginning of the study and after each intervention period. An untargeted metabolomics strategy using HPLC-QToF-MS followed by multivariate data analysis was applied to all urine samples.

Most compounds identified as being discriminant for cocoa consumption were related to theobromine and polyphenol metabolism, as well as to compounds produced during cocoa processing. In the case of the endogenous metabolites, the identifications suggested a reduction in the urinary levels of acylcarnitines and sulphation of tyrosine. These metabolites may be related to cardiovascular disease, although specific studies are needed to evaluate whether changes in these markers are a consequence of some metabolic alterations associated with cocoa intake, or are caused by decreased or increased regulation of some metabolic pathways that are affected by the consumption of this food product.
A Venn diagram was produced to display how many metabolites were characteristic of cocoa exposure in both studies [27, 28], together with those in another previous acute study that also used cocoa [29]. Fig. 3 shows that 10 metabolites were discriminant for cocoa consumption independently of the study design. These metabolites belong to the metabolic pathway of theobromine and to the microbial metabolism of cocoa polyphenols. These 10 metabolites were considered for the development of the multimetabolite biomarker model. The AUC values (95% CI) for the model were 95.7% (89.8–100 %) and 92.6% (81.9–100 %) in training and validation sets, respectively, whereas the AUCs for individual metabolites were <90%.

2. Replication of biomarkers of dietary exposure in nutritional studies with differentiated designs

There are practically no studies aimed at the replication of biomarkers of dietary exposure in populations in free-living conditions [30]. In this context, the replication of markers allows the level of evidence of observed associations to be increased, as previously suggested for genomic studies [31].

Diet control is a very important factor in the study of biomarkers of dietary exposure. It may have a high influence on the results, since the foods to which individuals are exposed during a clinical trial depend on dietary interventions or restrictions, or on the eating habits of individuals in the case
of observational studies [18]. This aspect has an important repercussion on the specificity and sensitivity of the candidate biomarkers. On the one hand, the markers identified in controlled intervention studies may not have sufficient specificity when attempting to apply them in observational studies because habitual diets may include other foods that also contain the same markers and that have been restricted during a nutritional intervention. On the other hand, when observational studies are being developed it should be taken into account that many foods are usually consumed together, following certain patterns of intake, which can lead to the identification of biased low-sensitivity markers [18]. Thus, the replication of a marker in studies with differentiated designs (in this case, controlled interventions and observational studies) is an indication that it is a metabolite with high specificity and sensitivity [11].

The studies summarized in this chapter have evidenced the replication of the discriminant metabolites of the metabolic footprint associated with the consumption of certain foods. Initially, markers of nut and cocoa consumption were characterized in controlled nutritional intervention studies [23, 25], most of which were also discriminant in a population analysed using an observational study design and taking into consideration their usual diet under free-living conditions [24, 26].

Another aspect to consider in the study of biomarkers is the type of biological sample used. Urine, along with plasma and serum, is one of the biological fluids most frequently used in nutrimetabolomics studies, and it has been shown to reflect a higher concentration of metabolites derived from food than plasma [18]. Twenty-four-hour urine samples have been described as a more robust method for monitoring daily dietary intake than the use of spot urine samples [12, 32]. The clinical trials with dietary interventions included in this chapter used 24-hour urine samples [23, 25]. However, collecting 24-hour urine samples is a difficult and cumbersome task, especially in large-scale epidemiological studies [32, 33]. Therefore, the replication of the exposure markers (initially characterized in 24-hour urine samples) in spot urine samples reinforces their discriminatory power independently of the type of sample used [24, 26].

Finally, the time course of excretion will define whether the compound is a short-, medium- or long-term marker. For example, in the study of urinary metabolome associated with habitual cocoa consumption, it was observed that metabolites that remained discriminant independently of the study design were those with an excretion pattern of at least 24-hours after ingesting the food, such as theobromine and polyphenol metabolites derived
from microbial metabolism [34, 35]. Therefore, these observations reinforce the concept that in observational studies, where subjects are evaluated under free-living conditions, biomarkers that are excreted later or during a wide time frame may be better predictors of food intake than those that are rapidly excreted [36, 37]. In contrast, short-term biomarkers will only be useful in those populations that consume the corresponding dietary source with some regularity and frequency [38].

3. Design of multimetabolite biomarker models to improve the prediction of dietary exposure

Given that most food constituents are widely distributed in several foods, very few compounds can be considered biomarkers of a particular food or food group. This would be the case of proline betaine for citrus intake [39] and resveratrol for wine consumption [40]. Additionally, it must also be taken into account that in some cases differentiated compounds, after undergoing various metabolic processes in the organism, may converge with common metabolites, as is the case for several groups of polyphenols in wine, which, after their absorption and metabolism, are usually transformed into common phenolic acids [41], or the different classes of ellagitannins that are found in walnuts, pomegranates and strawberries, which microbiota metabolize to common urolithins [42, 43].

In order to solve this issue, it has been proposed that by combining more than one compound in a multimetabolite model, a more precise measurement of consumption could be achieved. It was shown that generating models of biomarkers formed by more than one metabolite provided better results than with the measurements obtained for each individual compound [22, 24, 26]. These results reinforce the hypothesis that an improved discriminate dietary exposure ability is achieved through the use of biomarker models made of more than one metabolite. It is important to emphasize that these models are constructed of metabolites of different classes, most probably because each of these metabolites gives complementary information on dietary intake, whereas those that are left out of the model probably do not contribute any additional biological information to that which has already been part of the corresponding model.

To date, there have been very few cases in the scientific literature that have tried to work with combinations of nutritional biomarkers to improve the predictive capacity of dietary exposure measures [18]. One of the few examples that have been proposed so far is the ratio between two
alkylresorcinols for the consumption of whole grains [44]. However, in studies reported in this chapter a new proposal has been made that allows the consideration of ≥ 2 metabolites. This represents an important novelty in the field of nutrimetabolomics by opening an alternative route for the discovery of new biomarkers of dietary exposure.

4. Future perspectives

The application of metabolomics to the study of biomarkers of dietary exposure is still far from being exploited in depth. For example, most of the available untargeted nutrimetabolomics studies have been exploratory. Thus, for some markers there are very few studies with which to compare the results. For this reason, it is very important to continue replicating the markers in different populations and in studies with different designs.

Targeted studies on dose-response behaviours and interindividual differences are also needed before biomarkers can routinely be applied in nutritional studies. In fact, the maximum utilization of the data obtained through untargeted metabolomic studies is obtained when qualitative and quantitative analyses are combined, since the quantitative measurements of the markers using a targeted approach and in a controlled study improve its applicability and interpretation. Additionally, it will provide information that can be very useful in interpreting the metabolic pathways affected [45].

Some untargeted metabolomic studies have been performed to identify markers of the usual dietary patterns [46, 47], but the studies are too different in design and in the analytical approaches used. The results of these studies showed that dietary patterns are reflected in urine and plasma, although it is necessary to propose new studies focused on the determination of the inherent footprint of this food consumption to reinforce its predictive power.

The most recently proposed challenge includes the integration of different omic technologies (genomics, proteomics, transcriptomics and metabolomics) to obtain a more complete picture of health status and, thus, to unravel the links between disease prevention and dietary intake. Therefore, in future studies, the comprehensive understanding of dietary effects needs the approaches of systems biology, including genomics, proteomics, transcriptomics and metabolomics, combined with a suitable experimental design and a sufficient number of included subjects to be able to find the variables associated with the effects associated with the diet. These studies will require multidisciplinary working teams.
At the same time, it would also be interesting to analyse the relationships between classical health markers and biomarkers measured by untargeted metabolomics, with the aim of deciphering the biological connection between traditional clinical parameters and metabolic markers.

5. Conclusions

The main conclusion drawn from this work is that the application of an untargeted metabolomics strategy in the study of nutritional biomarkers enables the main differences in the urinary metabolome associated with dietary intake to be characterized. They are associated with food digestion, microbial metabolism and endogenous metabolism. Most of the biomarkers characterized in clinical trials of nutritional intervention have been replicated in individuals evaluated observationally in free-living conditions. The biomarkers that are usually replicated as discriminatory in studies with different designs (i.e. nutritional interventions and observational studies) are those that usually present a medium- and/or long-term urinary excretion with respect to the moment of ingestion of the corresponding food. The predictive ability of dietary exposure through mult metabolite biomarker models is greater than the ability of single metabolites when they are evaluated individually. The combined models could be extremely useful in improving accuracy during the evaluation of dietary intake.

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References