Nutritional Biomarkers: We Know What You Had for Dinner!

In 2005, 21% of English adults were classified as obese; this is expected to rise to 33% of men and 28% of women by 2010. 58% of type two diabetes cases and 21% of heart disease cases are thought to be related to excess fat, and obesity reduces life expectancy by an average of 9 years. Currently obesity costs the NHS £1bn a year, but recent warnings have hinted that it may cost upwards of £6.5bn, however, other consequences, such as obese people being unable to work, could cost a total of £45bn by 2050 [1,2].

The government maintains that parents do not recognise that their children are overweight; underestimate how much unhealthy produce they buy, and overestimate how much exercise their children do. In addition, only 38% of adults know that obesity can lead to heart disease, and 6% know about a link to cancer. These facts have spurred the government into a three year anti obesity campaign, called change4life which began with an £8 million television advertising campaign earlier this year [3].

However, whilst obesity or body mass index (BMI) could be seen as an excellent marker of a poor diet, the diet can affect long term health, without necessarily being correlated with a changing BMI. For example, it is possible for a person with a BMI in the normal region to have an unhealthy diet which would make them more susceptible to conditions such as heart disease and cancer.

> ***"It is estimated for example that around 30% of all cancers could be preventable via modifications to the diet."***

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So, why use a biomarker approach? Traditionally, research into the relationship between diet and disease state has been performed via the use of food diaries, particularly in epidemiological studies, which involve large numbers of participants. Unfortunately, people tend to over estimate the number of healthy food choices made and under estimate the unhealthy food choices. In the past this has hindered the search for associations between diet and disease. Biomarkers have the advantage in that they are independent of individuals having to note what they eat, and as such can offer an impartial picture of diet quality.

Examples of biomarkers include phospholipid fatty acids, which may be associated with intake of animal products and nuts, fish consumption, margarine and processed food (trans fatty acids). Vitamin B12 can be associated with meat and dairy intake, α and γ-tocophenol with vegetable oils and some fortified foods such as breakfast cereals, vitamin C, folate, and carotenoids with certain fruits and vegetables.

The picture is even more complicated when one takes into account variations in the individual's physiology and nutrient metabolism, as well as differences in adsorption of nutrients. For example in a feeding study of carotenoids in 79 male volunteers, absorption of pharmacologic doses of β-carotene had a coefficient of variation of 61%, although there was some evidence of within person consistency in response to a given load [4].

Given these variations, laboratory issues become even more critical in obtaining reliable biomarker data. Pre-analytical variations such as sample collection and stability must be taken into account. For example; folate may require a preservative such as vitamin C if the sample is to be stored long term and riboflavin is sensitive to light exposure [4]. Vitamin C is especially problematic, needing special storage and sampling conditions to obtain any meaningful results.

The laboratory quality control procedures also play a vital role in establishing new biomarkers and in establishing links between nutritional status and disease. Lack of control in the laboratory could in theory lead to misleading observations and consequently false conclusions. During studies, whilst the laboratory may have its own QC procedures, it is always useful to include blinded QC samples within the study by the investigator, increasing confidence in the data generated [4].

SUCROSE AND FRUCTOSE

Obesity is a major health problem in both the UK and US, and is frequently described as an epidemic. The cost to the health service is enormous, and if we take into consideration the growing evidence of a link between obesity and cancer, as well as type II diabetes, it is not hard to understand the increased impetuous by governments to highlight the importance of a healthy lifestyle and healthy eating.

People are often surprised by the connection between obesity and cancer; after all, the traditional image of someone with cancer is of a thin individual, as weight loss is a common problem for cancer patients. Epidemiological studies suggest a link between the intake of refined sugars and increased risk of several cancers, including colorectal, breast, pancreatic and endometrial cancer. However, proving a link between sugar intake and obesity has proven more difficult than we would perhaps expect, possibly due to the ambiguity of self reporting during dietary assessment. There is a tendency to under report foods that are considered unhealthy. In fact, when individuals have participated in national food surveys and are characterised by body weight, BMI is inversely associated with the percentage energy from sugars [5]. There is therefore little evidence that high sugars intake is associated with obesity. Recently, Tasevska *et al* have developed a method for the analysis of sucrose and fructose in urine that predicts usual daily consumption of total sugars [6].

Dietary intake of refined sugars cannot be derived from measurement of urinary glucose, however, it has been shown that a fraction of the sucrose and fructose ingested is excreted in the urine. Studies by Bingham *et al* [5] using this method applied to the EPIC Norfolk cohort of men and women recruited between the ages of 45 to 75 years, found no association between dietary intake of sugars and obesity. However, when taking the biomarker approach, by measuring the sucrose to fructose ratio, there was found to be a highly significant difference between normal weight and obese individuals (*Figure 1*).

Kuhnle *et al* have since gone further with their methods of analysis, developing both LC/MS and GC/MS methods [7], the GC/MS method having an advantage in that it can measure more compounds and possibly identify more biomarkers in one run, but sample preparation is laborious and, with a run time of almost an hour, sample throughput is compromised. Both the LC/MS and GC/MS methods compared well for the measurement of sucrose, and indeed it was shown that the LC/MS method could be used for the accurate determination of urinary sucrose and successfully differentiated between a low and high-sugar diet.

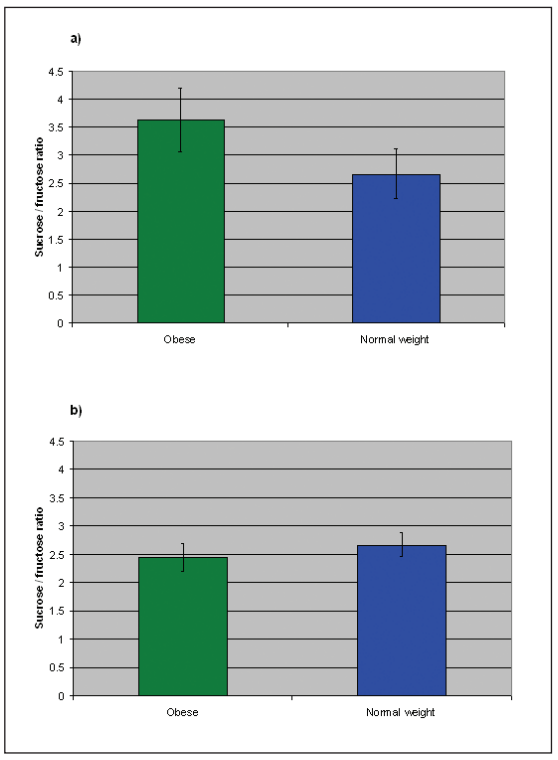

Figure 1. Sucrose fructose ratio for normal weight and obese people. a) biomarker approach, b) food diary data [5].

PHYTOESTROGENS

Phytoestrogens are diphenolic compounds that naturally occur in a variety of plants that can form part of the human diet. Interest in phytoestrogens stems from the belief that they may offer a protective effect against a number of human conditions including cardiovascular disease, certain forms of cancer, and menopausal symptoms. The two sub-classes of phytoestrogen which have been subjected to the most intense research are the isoflavones and the lignans. Isoflavones occur in foods such as legumes and in particularly high levels in soy products. Lignans are found in a wider variety of foods than the isoflavones, being present in grains, seeds, and in particularly high concentrations in linseed.

Analysis of phytoestrogens in biological fluids has been accomplished by time resolved fluorescence immunoassays, however, due to the large number of structurally similar metabolites, the use of more selective analytical techniques, such as GC-MS or LC-MS, is advantageous.

Whilst GC-MS has been successfully used in the quantification of phytoestrogens [8], in terms of robustness and quality of quantification we have found LC-MS/MS to be the method of choice [9]. Following addition of triply-^{13}C labelled analogues, to act as internal standards, samples are enzyme hydrolysed to cleave glucuronic acid and sulfate conjugates, thus forming the aglycones. Samples are then extracted using solid phase extraction. A relatively long run time of 14 minutes was required in order to achieve sufficient chromatographic separation of the large number of analytes from endogenous matrix effects. Clearly when utilising expensive equipment such as LC-MS/MS the run time is an important consideration.

One solution that we successfully applied to reduce the effective run time was to use column switching, with two analytical columns being employed, such that one column is re-equilibrated whilst another runs a gradient [9]. This allowed the run time to be decreased to 10 minutes, a significant and worthwhile improvement in throughput. However, a more effective approach was to make use of ultra performance liquid chromatography (UPLC), utilising sub-2 µm chromatographic media at elevated backpressures. This provided a significant increase in throughput with an analytical run time of 5.5 minutes. Even more impressive was the fact that even with the decreased analytical run time a matrix interference which co-eluted with one of the target analytes was clearly resolved using UPLC (*Figure 2*).

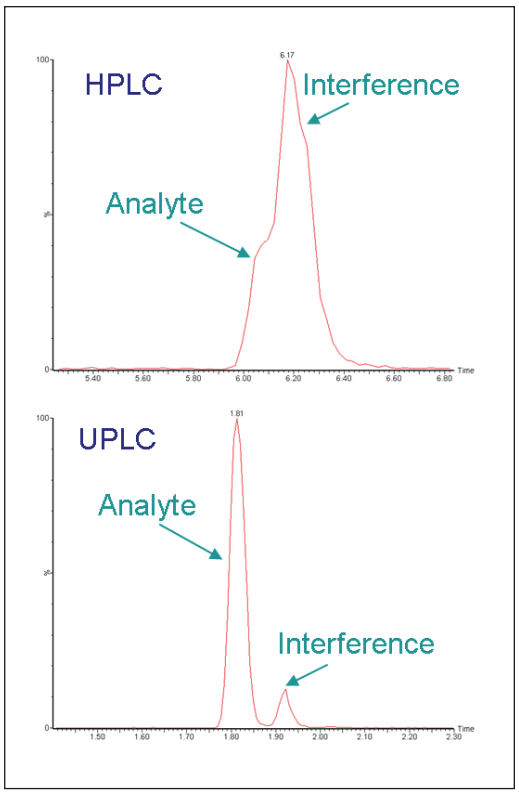

Figure 2. Resolution of a phytoestrogen matrix interference using UPLC-MS/MS.

Recently, LC-MS methodology developed in these laboratories was used in a study to quantify phytoestrogens in the plasma of over 1400 subjects [10]. The aim of the study was to assess the variability of phytoestrogen concentrations in plasma in European populations. Samples were analysed from 15 different regions across 9 different European countries, plus a 16th region (Oxford, UK) where participants were consuming a vegan or vegetarian diet. Mean concentrations of phytoestrogens were low, however, there was substantial variation between different regions. The mean level of the isoflavone genistein in the different regions studied is shown in *Figure 3*.

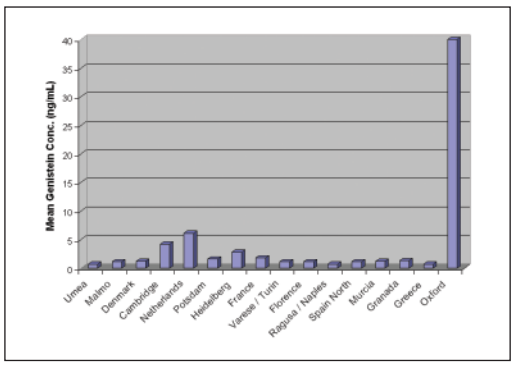

Figure 3. Mean level of genistein found across 16 different European regions. Note that the Oxford region includes a high number of vegan and vegetarian participants.

Excluding the Oxford subjects, it was found that isoflavone concentrations varied 8- to 13-fold and lignans 4-fold. Isoflavones in the Oxford subjects were 5 to 50 times higher than in non-vegetarian regions, which can be explained by the replacement of meat products by soy-based products. Region was the most important determinant of plasma concentration for all of the seven phytoestrogens measured. This is the largest study showing plasma phytoestrogen concentrations in healthy European people.

SUMMARY AND LOOK TO FUTURE ADVANCES

It is clear from studies carried out over many decades that diet has a significant impact on health. Whilst food records, in the form of diaries and food frequency questionnaires for example, have been shown to provide useful information in the study of diet and disease state, it is clear that these diaries can be subject to large measurement errors. What is even more concerning regarding the use of food diaries is that studies, such as the sucrose and fructose study mentioned in this article, have shown that not only does selective under-reporting of 'bad foods' occur, but that this selective under-reporting occurs to different degrees in different sections of the population.

An alternative to food diaries is nutritional biomarker measurements. Whilst these assays may be challenging to the analytical chemist, due to the low concentrations, requirements for high throughput, low sample volumes, and in some cases large numbers of analytes, the results are not subject to 'human bias' during data generation, at least not in any well controlled assay.

The use of modern instrumental techniques, such as UPLC, confers great advantages to the analyst when measuring nutritional biomarkers. The improved sensitivity, higher throughput, and possibly most notably the enhanced chromatographic resolution, can be vital to successfully measuring low concentrations in multi-analyte assays.

Whilst there are some clear advantages to the use of nutritional biomarkers, it is extremely important that these assays are well designed, well controlled, and validated. For example, it is important to have a good understanding of the biochemistry and kinetics of the analytes in question so that the results do not reveal a snapshot of the subject's last meal, but instead reflect the subject's dietary status over a period of time.

The importance of accurate, precise, well controlled analytical methods is crucial to the interpretation of results, as is the generation of accurate food diaries. Given that controlling the latter is difficult, the former becomes even more important. Unfortunately, since the analytical method is not the headline grabbing star of the research, and given that many researchers in the field are not analytical chemists, it is all too easy to relegate the analytical methods to the second tier.

So where will this area of research move to in the future? Well, nutritional metabolomics and nutrigenomics promise much but, as of yet, have not delivered any major breakthroughs. It is clear that our health is not solely affected by one or two components of the diet, but instead by many different components, in both a positive and negative way. To complicate the issue further, the effect of these different components of the diet may in turn be enhanced or reduced by other components. Therefore the use of multi-analyte studies to determine a number of nutritional biomarkers in relation to disease state is likely to become more important, whether this is accomplished via a metabolomics approach or, more likely, via a targeted approach to sets of biomarkers. The ultimate aim would be for us to be able to provide personalised dietary advice to individuals, in order to maintain not only short-term, but also long-term health.

Finally, a criticism that is often thrown at nutritional research by the general public is that 'one week we're told that something is good for us and the next week it's bad for us'. An outcome of the increased use of high performance, well validated and controlled nutritional biomarker assays, is that we may start to produce data that provides more consistent advice to the general public. Whilst the outcome of studies depends on a number of factors, having a robust and reliable assay in place undoubtedly reduces any errors involved and may provide the consistent advice on diet that the public desires.

Happy eating!

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