4.4. Preparing phytonutrient extracts

Different classes of phytonutrients need different extraction methods to prevent losses during and after their extraction. Generally, aliquots of the (freeze-dried or still frozen) food powder are instantaneously mixed with an appropriate extraction solvent to inhibit chemical reactions and inactivate interfering enzymes and other proteins as far and quickly as possible. Depending on the phytochemical compound and material to be analysed, antioxidants like butylhydroxytoluene (BHT), butylhydroxyanisole (BHA), vitamin C (if this latter is not the target in the analysis), oxygen-depleting substances like sodium bisulphite and redox-metal (e.g. Fe, Cu) chelating agents like ethylene diamine tetra-acetic acid (EDTA) may be needed. These are usually added in excess to the extraction solvent. Once mixed, the phytonutrient extraction can be performed in an ultrasonic bath or other apparatus that can well disrupt the plant cells to release the nutrients. A centrifugation or filtration step is then applied to remove the proteins, cell walls and other debris from the food-solvent mixture. The resulting clear extract may be used directly, or further purified and concentrated if needed for the specific analysis. Extraction methods are usually validated by parallel extraction of material with and without the addition of a fixed amount of authentic standard to the extraction solvent; this is called standard addition. Then, the recovery of the standard added is determined, by calculating the ratio between amount originally added and the amount recovered after extraction: recovery should preferably be 90% or better. Extraction methods may be separately optimized for different plant species and tissues, in view of differences in their composition (e.g. level of endogenous antioxidants) or pH-buffering capacity (stability of most phytonutrients is low at high pH).

Lipid-soluble phytochemicals like **carotenoids** (vitamin A) and **tocopherols** (vitamin E) can be well extracted from frozen (water-containing) powdered food using an organic solvent like a mixture of chloroform and methanol, which also induces protein denaturation and precipitation. By subsequently adding equal amounts of water or an aqueous buffer, the organic phase containing the lipid-soluble nutrients will be separated (2-phase separation) and the organic layer can be collected for the actual analysis (Moco *et al.* 2007). BHT is usually added to all extraction solvents to prevent oxidation of these lipid-soluble phytochemicals.

Water-soluble phytochemicals like **vitamin C** (Moco *et al.* 2007) and **phytate** (Zhang and Bai 2014) are extracted using a strong acid that both dissolves and preserves these acidic phytonutrients while denaturing proteins. After centrifugation to clarify the supernatant, the extracts may be directly used for their analysis directly. If there is too much interference by co-eluting compounds, depending on plant species and tissue, a clean-up step to remove those interfering compounds may be needed. Solid phase extraction (SPE) with different types of bonded phases, depending on polarity and charge of nutrients, is commonly used to clean-up crude plant extracts. However, as such clean-up step may result in compound losses, recovery needs to be checked using authentic standards of reference compounds. The water-soluble vitamin **folic acid** is not present in plants as such; instead plants contain a range of water-soluble, polyglutamated and reduced folate forms. These folates are present at relative low concentrations. An enzymatic digestion of the food proteins and carbohydrates is required for their optimal release; antioxidants such as ascorbic acid and mercaptoethanol are usually added to prevent loss of the labile folate forms (Lawrance 2014). If a full folate profile analysis is required, e.g. for bioavailability studies or clinical research purposes, all (main) folate forms present in the plant sample should be identified and quantified. Such detailed analysis may not be needed for the estimation of total folate activity of foods; then it is only necessary to ensure that the folates are well extracted and converted into a form that can be well detected. For such total folate analysis, the polyglutamate chain is usually cleaved during extraction by adding excess of specific enzymes, in order to simplify subsequent detection of the resulting mono-glutamate form.

Phenolic compounds including flavonoids show intermediate polarity between polar and apolar: they are so-called semi-polar compounds. These can be well extracted in an acidified aqueous-organic solution, like 75% methanol (MeOH), ethanol or isopropanol in water, containing 0.1% formic acid or acetic acid as acidifier. The amount of acidifier may be adapted to compensate for the pH-buffering capacity of the food material. The organic component of the extraction solution both enables extraction of the phenolic compounds and precipitates oxidizing enzymes and other interfering proteins; the acidic environment (~pH 3) prevents losses by chemical oxidation and aspecific binding (hydrogen-bonds) of the phenolic groups in this class of phytochemicals to the precipitating proteins (Moco *et al.* 2006). If total levels of flavonoids rather than levels of the individual flavonoid structures are required, the food material may firstly be hydrolysed by adding a strong acid such as 1.2 M HCl and heating the mixture at 90°C, in order to remove the glycosidic decorations on the flavonoids (Hertog *et al.* 1992). The flavonoids are then analysed as their aglycons, rather than their various decorated forms as originally present in the plant. Since different plants may contain different flavonoid glycoside structures, the hydrolysis procedure needs to be optimized for each plant material. This acid hydrolysis works for the majority of crops, as most of them accumulate flavonoids as so-called *O*-glycosides (carbon-oxygen linkage). However, the less common *C*-glycosylated flavonoids (carbon-carbon linkage), present in e.g. barley grains, citrus, dates, rooibos tea and some ethnopharmaceutical herbs (Courts and Williamson 2015) cannot be hydrolysed, neither chemically nor enzymatically. Hence, for these *C*-conjugated flavonoids the only possibility to analyse them as their intact molecules as present in the plant, and to quantify each structure separately using authentic standards or appropriate reference compounds.

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