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Physiochemical Characterization of Nutraceuticals

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CHAPTER 6

Physiochemical Characterization of Nutraceuticals

Ajoy Koomer

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INTRODUCTION

According to the American Nutraceutical Association, the term “nutraceutical” was derived by condensing the terms “pharmaceutical and nutrition” in 1989 by Stephen DeFelice, who was founding chairman of the Foundation for Innovation in Medicine [American Nutraceutical 2008]. DeFelice suggests that, “A nutraceutical is any substance that is a food or a part of a food and provides medical or health benefits, including the prevention and treatment of disease. Such products may range from isolated nutrients, dietary supplements and specific diets to genetically engineered designer foods, herbal products, and processed foods such as cereals, soups and beverages” (2008). Nutraceuticals and dietary supplements can be broadly classified into five categories, namely vitamins, minerals, botanical substances, herbal extracts, and miscellaneous or specialty components [Krull and Swartz 2001]. The vitamins category includes fat and water-soluble vitamins and nutritional factors [Krull and Swartz 2001]. The minerals category comprises mineral chelates, salts, single
and trace elements, and multiple minerals consisting of amino acids mixes [Krull and Swartz 2001]. Botanical substances include mixed and single whole herbs, essential oils, tea mixtures, and traditional formulas [Krull and Swartz 2001]. The specialty components include antioxidants, carotenoids, essential (omega-3) and omega-3 and -6 fatty acids, phytosterols, anthocyanins, flavonoids, probiotics, lecithins, glandular, diet acids, and digestive acids [Krull and Swartz 2001]. As noted by specialists, nutraceuticals are gaining public acceptance because of escalating consumer market share for “wellness products” [Krull and Swartz 2001; Dureja, Kaushik, and Kumar 2003; Metha et al. 2007]. Because of a lack of standardization of active ingredients in nutraceutical-related products coupled with increased market interest, the United States has had increased attention “in the marketing, claims substantiation, manufacturing, and FDA-based regulations of nutraceuticals” [Krull and Swartz 2001; Dureja, Kaushik, and Kumar 2003; Mehta et al. 2007].

The tightening of the regulation apparatus is the driving force for the implementation of reliable analytical techniques for the reliable detection of active gradients in nutraceutical complex matrices and their physicochemical characterization for enhanced formulation and quality-control studies [Krull and Swartz 2001; Dureja, Kaushik, and Kumar 2003]. The nutraceutical testing methods available in industry include stability testing, dissolution testing, in vitro release rate testing, content uniformity testing, high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC), mass spectrometric analysis, Fourier transform infrared spectroscopy, and ultraviolet/visible light (UV-vis) spectroscopy [Analytical Solutions 2008]. A major factor in physicochemical characterization is stress and stability testing. Stress testing is performed to identify all potential degradants. Generally, chromatographic techniques are used as methods of validation in stress testing, with detection being performed by UV-vis spectroscopy [Mehta et al. 2007]. In this chapter, we will focus on physicochemical characterization of phytosterols, fatty acids, carotenoids, amino acids, anthocyanins, and water-soluble vitamins.

**PHYTOSTEROLS**

Phytosterols are diphenolic compounds present in plant and animals that resemble the human sex hormone estrogen [Hurst 2002]. They can be classified into three categories, which are coumestans, lignans, and isoflavonoids [Hurst 2002]. They can be detected and quantified by TLC, HPLC, and gas chromatography-mass spectrometry (GC-MS) coupled with UV-vis spectroscopy [Hurst 2002]. However, before implementation of any methods for validation, sample preparation and extraction play a critical role. Usually, phytosterol matrix samples are spiked with 20% (w/v) of purified isoflavones, such as daidzein, genistein, and biochanin A [Hurst 2002]. The spiked solution consists of 1.3% (w/v) of tertiary butyl hydroquinone in methanol [Hurst 2002]. The use of isoflavones as internal standards stem from the pros that it allows for accurate “determination of analyte recoveries and account for weight loss in the extraction procedure” [Hurst 2002]. After sample preparation, they are extracted with 80% methyl alcohol for 0.5 h, although other solvents, such as ethanol, acetonitrile, and acetone, can be used [Hurst 2002]. TLC has been used routinely in the identification
of isoflavonoids [Hurst 2002]. Scientists have used precoated polyamide TLC plates for the separation of isoflavones and other diphenolic compounds from soya bean extracts [Hurst 2002]. As noted by scientists, the methanolic extracts spotted on 20 × 20 plates were developed with methanol/acetic acid/water mixture in proportions of 90:5:5 (weight/volume) [Hurst 2002]. After eluting the bands with ethanol, they were “rechromatographed on polyamide using chloroform-methanol-methyl ethyl ketone solvent system in the ratio 12:2:1” [Hurst 2002]. When viewed under a UV lamp at 366 nm, the analyzed fractions produced characteristics Rf values corresponding to daidzein, genistein, formononetin, and biochanin A [Hurst 2002]. It is to be noted that TLC is a qualitative procedure that cannot be used for quantification of individual phytosterol fractions from complex plant and food matrices [Hurst 2002]. HPLC is the method of choice here. Although MS techniques can be used with improved sensitivity, they are not popular because of cumbersome sample preparation time and costs [Hurst 2002]. Generally, phytosterols can be purified and quantified by mixtures of “methanol or acetonitrile and aqueous acids or buffers” by the use of reverse-phase (RP) C18 separation columns [Hurst 2002]. The most frequently used HPLC techniques use either linear or nonlinear gradient elutions for isoflavonoid separation and “quantitative estimation from legume or soybean matrices” [Hurst 2002]. Generally, in gradient elution HPLC, “acetonitrile increases by 2.25% min,” enabling “the separation of isoflavone-β-glucoside conjugates and aglucones” in a single experiment [Hurst 2002]. The experimental runtime usually does not exceed 60 min, with equilibration between the cycles [Hurst 2002]. Recently, RP-HPLC has been used to separate isoflavones such as daidzein and genistein [Hurst 2002]. Isocratic elution conditions have been reported by researchers in the separation of phytosterols, although the method has been unsuccessful, except for genistein [Hurst 2002]. Because of the high concentration of isoflavonoids in soy or legume products, UV-vis spectroscopy coupled with RP-HPLC is used for detection [Hurst 2002]. However, the method suffers from challenges in quantifying isoflavonoids from sources with low concentration or if phytosterols other than isoflavonoids are involved [Hurst 2002]. In those cases, “fluorometric detection, amperometric methods, or thermospray MS with SIMI” can be considered to increase sensitivity of common UV detection systems [Hurst 2002].

FATTY ACIDS

This section focuses on quantitative functional analysis of essential omega-3 fatty acids, namely EPA, DHA, and ALA [Hurst 2002]. For the quantitative estimation of fatty acid profile in complex matrix (functional foods), the usual steps are extraction to release the free fatty acids, derivation of the released fatty acids, and chromatogram analysis with GC with flame ionization detection, although other techniques such as GC-MS and HPLC have been used with success [Hurst 2002]. The Folch method of lipid extraction, using chloroform and methanol as solvents in the volume ratio of 2:1, is most common [Hurst 2002]. To eliminate the nonlipid contaminants such as carbohydrates and amino acids, the components chloroform-methanol-water/0.88% potassium chloride must be adjusted to 8:4:3 by volume, yielding a biphasic system
The free fatty acids are then usually derivatized to fatty acid methyl esters (FAMEs) [Hurst 2002]. These derivatives are volatile and render themselves to excellent gas chromatographic analysis [Hurst 2002]. In GC, the area percentage of each FAME peak is proportional to the weight percentage of each FAME [Hurst 2002]. However, this method overestimates long-chain fatty acids and underestimates short-chain ones. Neglecting this caveat, this is still the most commonly used procedure [Hurst 2002]. The quantification of individual FAME in milligrams per gram of the total sample is facilitated by the use of an internal standard, which can be added during either extraction or derivatization process [Hurst 2002]. Recently, GC-MS and HLPC techniques to characterize DHA and ALA have been reported [Hurst 2002].

CAROTENOIDS

Carotenoids are lipophilic compounds present in both plant and animal species. The unique chromophore for these compounds containing polyene structures make amenable to light absorption in the visible range; hence they are identified by UV-vis spectroscopy [Hurst 2002]. Colors usually vary from yellow to red via different shades of orange. The most common techniques used for quantitative identification of carotenoids from plant matrices involve TLC/UV-vis spectroscopy or HPLC/UV-vis spectroscopy. Different carotene components can be separated using TLC following the method of Gross [Hurst 2002]. Silica gel is conventionally used to prepare the stationary phase in chromatographic separation with low polarity solvents used as eluents. After separation in TLC plates, the bands corresponding to each pigment are recovered from the chromatographic support and eluted with low polarity solvents, such as acetone. Filtration is used to remove the adsorption materials, and the absorption spectrum of the pigments is quantified using the Lambert–Beer law. The actual quantification of carotenes in the sample is then performed, knowing the following parameters in the hand: “weight of sample extracted, final volume of extract, volume of extract chromatographed and volume of elution of pigments chromatographed” using a modified equation based on the Lambert–Beer law [Hurst 2002]. Both normal-phase and RP-HPLC coupled with visible spectroscopy have been reported for the quantitative estimation of carotenoids. The former uses a polar compound in the stationary phase, whereas the latter uses nonpolar stationary phases such as octa silane or octadecyl silane. If fixed wavelength detector is used, the selected wavelength is 450 nm; for diode arrays or multiple wavelength detectors in UV-vis, spectra are obtained at the absorption maxima of each pigment [Hurst 2002].

ANTHOCYANINS

Anthocyanins are a diverse group of water-soluble pigments responsible for the red, blue, and purple colors of plants. They are all characterized by the cyanidin aromatic ring structure and are classified by the number of sugars and their position in the aglycon chain [Hurst 2002]. Andersen and his colleagues at the University
of Bergen in Norway developed analytical techniques for the isolation, separation, and characterization of anthocyanins. In the initial step, materials were extracted in methanolic solution containing 1% trifluoroacetic acid, followed by partitioning the extract against ethyl acetate to eliminate the flavonoid contaminants [Hurst 2002]. The partitioned mixture was then subjected to column chromatography using an Amberlite XAD-7 column, eluted with 50% methanol and 100% methanol containing trifluoroacetic acid, subjected to fractionation in a Sephadex LH 20 column, and further eluted with methanol, water, and trifluoroacetic acid mixture; it is then subjected to additional purification by preparative HPLC using a RP C18 column. The purified compounds are detected by UV-vis adsorption spectroscopy based on absorption maxima of individual aglycon units. For example, peonidin-3-glucoside exhibits absorption maximum between 520 and 526 nm whereas delphinidin shows the same between 532 and 537 nm. Recently $^1$H and $^{13}$C nuclear magnetic resonance spectroscopy have been used to detect anthocyanins [Hurst 2002].

**AMINO ACIDS**

Of more than 200 amino acids found in nature, about 20 are components of proteins including enzymes. Of the 20 amino acids, nearly half of them are essential amino acids. These amino acids cannot be synthesized by humans and had to be obtained as nutritional supplements [Hurst 2002]. A nutritionist generally focuses on obtaining amino acid profile for the essential amino acids. The physicochemical characterization of amino acids involves acid or alkaline hydrolysis of the samples and extraction, followed usually by HPLC purification and detection. However, detection of amino acids poses challenges because only three of them, phenylalanine, tyrosine, and tryptophan, have significant UV absorption. Also, only tyrosine and tryptophan fluoresce. Thus, the universal detection techniques stress on refractive index and light scattering or derivatizing the amino acid to an intermediate product that shows absorption in the UV-visible range of the spectra” [Hurst 2002]. This derivatization can be performed precolumn or postcolumn. Precolumn indicates before HPLC separation whereas postcolumn refers to after HPLC purification. It should be noted that each procedure has from pros and cons. The groups commonly used in precolumn derivation include 9-fluorenylmethyl chloroformate and $o$-phthalaldehyde. In postcolumn derivatization, typically ninhydrin or and $o$-phthalaldehyde is preferred. When using postcolumn derivation, ones uses ion exchange chromatography, which separates amino acids based on charges [Hurst 2002]. Also, in this case, tedious sample preparation is not required. In cation-exchange chromatography used for amino acid purification, the stationary phase is negatively charged and traps the positively charged amino acid at a low pH. On the contrary, when precolumn derivatization is preferred, RP-chromatography is used with either C8 or C18 on silica. The added advantage stems from the fact that silica supports can stand high pressures compared with polymeric supports used in exchange chromatography with increased flow rates and reduced runtimes. The major drawback with precolumn derivatization is the chance of sample manipulation before HPLC separation and purification. The cons of the postcolumn method include the fact that adding reagents to the eluant can adversely interfere with the chromatogram.
resolution [Hurst 2002]. Also, the postcolumn reactor is costly and derivatization is restricted by reaction kinetics and the chemical compatibility of the introduced group with mobile phases of the chromatograph [Hurst 2002]. Thus, choosing the best method will depend on the sample properties and reaction conditions.

**WATER-SOLUBLE VITAMINS**

As the name suggests, vitamins are “vital amines” that play an important role in human growth and development. Since the past decade, vitamins have drawn attention because of their nutritional implications and the need to develop standardized quality-control procedures [Hurst 2002]. However, development of physicochemical methods for characterization is difficult because these compounds are diverse in nature, present in low quantities in complex matrices, such as functional foods and vegetables, mimicking of their activity by other compounds, and the special stability considerations. Most of the analytical methods described in literature focus on extraction, purification, and detection [Hurst 2002]. The extraction procedure from the complex matrices may involve heat, acid or alkali or enzymes, followed by cleanup procedures, and then quantitative estimation by RP-HPLC coupled with UV, fluorescence, or protein binding. The water-soluble B vitamins, such as thiamin, riboflavin, and niacin, may be estimated by RP-HPLC coupled with fluorescence or UV absorption. For thiamine, after extraction, it is convenient to convert it to fluorescent thiocrome by oxidation and then do quantitative separation and identification by RP-HPLC coupled with fluorometry using an excitation wavelength of 360–365 nm and an emission wavelength of 460–480 nm [Hurst 2002]. For RP-HPLC, usually C8 or C18 columns are used with the mobile phase ranging from organic solvents and ion pairs to organic-aqueous buffer mixtures. It should be noted that ion exchange and normal HPLC purification has also been reported for thiamine and other B vitamins. Cobalamin or vitamin B_{12} can be characterized by HPLC coupled with specific protein binding assays involving radio isotopes or enzymes. Its diversity and low propensity in food matrices make it an unsuitable candidate for UV detection; the same applies to biotin or folic acid [Hurst 2002].

**REFERENCES**


