Food Chemistry, Function and Analysis

Nutraceuticals and Human Health

The Food-to-supplement Paradigm

Edited by Paul A. Spagnuolo

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Nutraceuticals and Human Health
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Edited by

Paul A. Spagnuolo
University of Guelph, Canada
Email: paul.spagnuolo@uoguelph.ca

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Preface

On my first day as an Assistant Professor, I was tasked with designing a brand new course. With absolutely no idea or training on how to do that I thought – there must be a textbook on the subject. Long story short, I did not find what I was looking for. Fast forward several years later and now an Associate Professor at a different institution, I still find myself looking for that textbook. Having been co-instructor for an upper-year course in *Functional Foods and Nutraceuticals* for several years now, I constantly find myself scrambling to find relevant, high-quality and up-to-date research that will help to explain the convoluted and often frustrating world of dietary-supplements/nutraceuticals. This drove me to put together that elusive book that I had been searching for.

I wanted to do something that covered all aspects of nutraceuticals. I wanted it to be more than just a resource that helps to uncover the fundamental question that those of us who take supplements ask – will it actually help me? Or, in more appropriate terms, what is the evidence that supports its use? While fundamental and overarching, this particular question often neglects a number of facets of this topic and overlooks key areas that are central to the quality of the actual product. Research summarizing whether a product performs according to the purported claims without question has merit, but overlooks how the product goes from plant/food-to-pill, how processing can alter bioactive structure and function, the regulations around claims and development, and what happens when that pill finally gets consumed. All of these factors play a crucial role in answering that fundamental question. Thus, the purpose of this book wasn't just to help me find that book, but was also to create an ultimate resource that will help explain the full process by which nutraceuticals go from plant or food to a health-improving pill.

Paul A. Spagnuolo

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Dedication

To:

My nonni: Gennaro & Maria and Michele & Maria whose love, courage and sacrifice enabled the opportunities afforded to me today. To my parents and sister: Susan, Anthony and Laura who relentlessly supported me every step of my journey.

For:

My wife and children, Christina, Jake and Siena who remind me every day of the importance of love and laughter and my true purpose.

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CHAPTER 1

Nutraceuticals

PAUL A. SPAGNUOLO

Department of Food Science, University of Guelph, 50 Stone Rd.,
Guelph Ontario, Canada N1G2W1
Email: paul.spagnuolo@uoguelph.ca

1.1 Introduction

Food-derived bioactive compounds are commonly referred to as nutraceuticals (in Canada) or dietary supplements (in the United States (US)). They are defined primarily by two features: (1) their ability to impart cellular or physiological effects and (2) their form, which is independent of the conventional product in which they are found (*i.e.*, as a powder or pill). In the US, dietary supplements are classified as foods and regulated by the Dietary Supplements Health and Education Act (DSHEA); while in Canada, they are defined under the umbrella of natural health products (NHPs) and are regulated as a subset of drugs under the Natural and Non-prescription Health Products Directorate (NNHPD). They are recognized as self-care products, making them available over-the-counter and considered safe for use without direct oversight of a health care practitioner. With this accessibility and consumers' desire to improve their own health care, patients frequently consume these products.

Nutraceutical was a term first coined in 1989 and is a hybrid word derived from nutrition, which is the science of food and nutrient effects on the body, and pharmaceutical, which is a compound manufactured for medical purposes. Health Canada defines nutraceuticals as ''a product isolated or purified from food that is sold in a medicinal form that is not associated

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with food and is demonstrated to have a physiological protective effect against chronic disease''.[1] Interestingly, this definition omits the utility of these products as potential therapeutics for disease. While the debate rages over the quality of the scientific evidence, which is commonly misinterpreted by media and overstated by corporations that market these products, undoubtedly there is a place for ''bioactive molecules with demonstrated physiological benefit'' (*i.e.*, words directly extracted from Health Canada's definition) for use as disease therapeutics. The medical research and/or regulations simply need to catch up with consumer demand for these compounds.

1.2 Nutraceuticals for Deficiency

The National Institutes of Health (NIH) recommends that if you do not eat a variety of nutritious foods, then ''some supplements might help you get adequate amounts of essential nutrients''.[2] They further state, before recommending following the food guide, that ''supplements can't take the place of the variety of foods that are important to a healthy diet''. Indeed, a general belief exists that supplementation is only useful in periods of deficiency and, historically, there is no denying this observation. Vitamins C or D to prevent scurvy or rickets, respectively, are classic examples of the utility of nutrition to prevent disease. Similarly, folic acid supplementation to prevent spina bifida has been implemented with great success; however, it should be noted that supplementation recommendations are implemented because fewer than 1 in 3 women of child-bearing age consume the recommended dose.[3] These are clear examples of how supplementation can prevent diseases in vulnerable populations, but can bioactive molecules be used to prevent or treat chronic disease in populations that are not vulnerable? Does an invulnerable population actually exist?

1.3 Why are Nutraceuticals Needed?

Humans require food to survive and our evolutionary history is filled with agricultural advancements that have resulted in the stable production of food free from harmful pathogens. These successes, however, have paradoxically come at a cost of these processed foods being of lower nutritional quality. For example, whole grains such as wheat and rice contain three layers: bran, endosperm and germ. The bran layer is comprised of minerals and B-vitamins, which are important enzymatic co-factors, and fiber-rich materials, which provide physiological benefits ranging from improving digestion, regulation of cholesterol and glucose absorption. The germ layer consists of phytochemicals, B-vitamins and vitamin E whereas the endosperm consists primarily of carbohydrates and proteins. With the advancement of agriculture, development of new preparation and storage techniques, and the advent of the industrial revolution, whole grains became

highly processed. Milling separates the bran and germ leaving behind only the endosperm. Further refinement results in a powder (*i.e.*, flour) that is suitable for long-term storage and can, in-turn, be used to create a wide range of products. However, during that process, all of the fiber is lost and between 70 and 80% of the vitamins are also lost.[4] This drastic reduction in nutritional quality has resulted in flour being fortified with B-vitamins;[5] calcium and iron may also be added as a means of increasing the nutritional value of flour-based products. This classic example demonstrates how processing alters nutritional quality and has directly resulted in the need to re-introduce bioactive food compounds. While fortification has become the solution to maintain the nutritional value of wheat, other products that undergo similar processing may not be as fortunate. Moreover, highly processed and foods of convenience, which make up nearly half of our daily total caloric intake, further add to the diminished quality of the food supply.[6] Couple processing with the drastic alterations in the diversity of our food supply that has favored, instead of more nutritional varieties, cultivars resistant to fluctuations in growing conditions and that better survive harvesting, storage and transport, and the overall quality of our food supply is diminished.

Food guides are created by governments with the intention of improving population health and minimizing disease. Offering guidance on appropriate food selection choices, these documents are common across the globe (Table 1.1). However, whether the population believes in or follows the guide is debatable. A 2019 Canadian survey showed that only 49% of people believe that the food guide is an important document and 52.4% say that they face barriers, including economic and cultural, in following the guide. This calls into question whether these expert recommendations are practiced, which can greatly impact the overall health of the population. While increases in chronic disease burden is not the ideal indicator to link disease onset with a lack of adherence to a food guide, it certainly speaks to the health, or lack thereof, of the global population.

Together, these examples show that a diminished quality of the food supply and general non-compliance with expert recommendations of following food guides are contributing factors leading to poor population health that likely drive consumers toward the use of supplements.

Table 1.1 Global distribution of countries with a food guide.

Continent	Countries with a food guide (%)
North America	87
South America	67
Africa	11
Europe	61
Australia and Oceania	14
Asia	35

1.4 Supplement Use

According to a recent survey, nutraceuticals are consumed mainly to ''improve'' or ''maintain'' health.[7] Whether this is: (1) to combat the reduced quality of the processed food supply, (2) a result of a more health-conscious consumer, (3) a result of a booming global billion-dollar supplement industry, or (4) a combination of all three, nutraceuticals are popular compounds consumed by 70–75% of the population. Moreover, and far more concerning, is the alarming statistic that up to 69% of prescription drug users did not report dietary supplement use to their physician.[8] As such, regardless of your position on the value of these products, they are readily available and consumed with great frequency.

Nutraceuticals fall under the broader category of NHPs, which is a global industry estimated to grow to over $360 billion (USD) by 2020. Natural products, which have contributed greatly to medicine, are derived from various sources including microbes, marine organisms, food and terrestrial plants. From 1981 to 2014, approximately 51% of all Food and Drug Administration (FDA)-approved drugs were natural products or their derivatives. Morphine was originally isolated from opium resin and quinine from the chincona tree. Indeed, acetylsalicylic acid (ASA), better known as aspirin, is a chemical modification of salicin, which was originally derived from the bark of the willow tree. Among the 141 approved anti-bacterial agents, 73% are derived from natural products.[9,10] Further, 83% of the FDA-approved chemotherapeutics are from natural sources.[9] Among these are paclitaxel and irinotecan, which are the cornerstones of many solid tumor drug regimens and were originally isolated from the bark of the Pacific yew and trees of the *Camptotheca* genus, respectively. In addition, nucleoside analogues such as cytarabine and decitabine, which are used to treat hematological malignancies, are synthesized to mimic structures found in marine sponges. Natural products also influence the discovery of new molecular entities (NMEs), making up 38% of the FDA-approved NMEs and 22% of approved drugs in 2014.[9,10] It is evident that research on natural products has been and continues to be essential in drug discovery, a fact typically overlooked by critics of natural product research.

1.5 Product Development Considerations

Up to this point, we have focused mainly on the finished product. However, in the process of nutraceutical development (Figure 1.1), there are many steps that can drastically change the nature and quality of the final product. While the final product dictates the clinical findings that determine the overall value and claims that can be made, final product variability is dependent on several factors. The nature and quality of the starting material (see Chapter 3), the nature of the extraction process (see Chapter 4) and methods used to formulate (or encapsulate) the product (see Chapter 6), all together determine the concentration and structural integrity of the

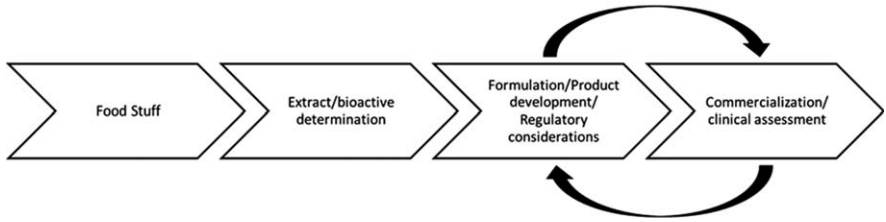

Figure 1.1 A simplified schematic diagram of the nutraceutical product development process.

bioactive within the final product. Without taking these factors into consideration, questions about quality as they pertain to standardization, dosing, stability, bioaccessibility and bioavailability of the bioactive can easily be overlooked. As outlined within several chapters of this book, all of these factors must be *first* considered when determining the value of the final product, as each can impact the safety and efficacy of the nutraceutical.

1.6 Conclusion

There is a great history of nutraceutical use for the management of disease and a place in modern and future medicine for these compounds. However, there is a glaring need for more research to ensure the safety and efficacy of these products and more awareness of the multiple steps that go into product development. Extreme caution should be used when consuming supplements. It is often wrongly stated or assumed that these products are safe because they are derived from nature and/or food. This is simply not true and is discussed further in greater detail in Chapter 5. Thus, it is imperative to continue dialogue and research on the benefits and potential negative effects of supplement use. As supplements continue to increase in popularity, innovation and knowledge become more and more important to ensuring overall population health.

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CHAPTER 2

Regulation of Nutraceuticals in Canada and the United States

PAULA N. BROWN,*[a] MICHAEL CHAN,[a] JOSEPH M. BETZ,[b] MOHD SHAHID,[c] SHAWN CANNON,[c] JESMY PALISSERY[c] AND MELANY P. PUGLISI[c]

[a] Natural Health and Food Products Research Group, British Columbia Institute of Technology, Burnaby, BC, Canada V5G3H2; [b] National Institutes of Health, Office of Dietary Supplements, Bethesda, MD 20892, USA; [c] Chicago State University College of Pharmacy, Chicago, IL 60423, USA
*Email: paula_brown@bcit.ca

2.1 Introduction

The intent of this chapter is to provide a general overview of the regulation of nutraceuticals in Canada and the United States. The term nutraceutical is used frequently to describe a wide variety of products on the market. The Oxford English Dictionary provides one definition that describes a nutraceutical as ''A foodstuff, food additive, or dietary supplement that has beneficial physiological effects but is not essential to the diet''.[1] Yet, this definition is not recognized by any regulatory authority in North America. Indeed, under regulatory systems that are primarily based upon explicitly defined product categories, the term nutraceuticals does not exist to describe any product category.

Under the regulatory perspective for both jurisdictions, a nutraceutical would first need to be categorized into a defined product category prior to its

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ultimate regulatory fate being decided. Given the wide spectrum of products that can fall under the umbrella of nutraceuticals, no single regulatory pathway in either the Canadian or United States regulatory systems is capable of addressing every nutraceutical product. To accomplish the goals of this chapter it is thus necessary to discuss the regulations covering several different defined product categories into which nutraceuticals could fall within the Canadian and the United States regulatory systems. This chapter will first discuss the Canadian regulatory framework, followed by the United States regulatory framework.

2.2 Overview of Nutraceuticals Regulation in Canada

In Canada, both food and drug products are regulated by Health Canada with various directorates and branches overseeing specific product categories.[2] The various definitions attributed to functional foods and nutraceuticals impart to them properties associated with both foods and drugs. As such, a particular nutraceutical may be considered a food, a drug or a Natural Health Product (NHP) under Canada's regulatory system. The category into which a nutraceutical would fall is very much dependent on its ingredients, history of use, associated risks, and presentation.

Generally, the first classification criteria for such products centers around whether or not a therapeutic claim is being made for the product. Under the Food and Drugs Act, a drug is defined as ''any substance or mixture of substances manufactured, sold or represented for use in the diagnosis, treatment, mitigation or prevention of a disease, disorder or abnormal physical state or its symptoms, in human beings or animals, restoring, correcting or modifying organic functions in human beings or animals, or disinfection in premises in which food is manufactured, prepared or kept''.[3] As such, any nutraceuticals making a therapeutic claim are considered drugs. These products are subject to drug law and their safety, efficacy and quality are assessed prior to being given market authorization, although there are exceptions for products meeting certain conditions to be regulated as foods. Those products not making a therapeutic claim would be considered foods under the Canadian regulatory system.

2.2.1 Regulation of Nutraceuticals Considered Food Products

Food products are subject to the relevant sections in the Food and Drugs Act[3] and regulations.[4] The Food Directorate within Health Canada[5] and the Canadian Food Inspection Agency (CFIA)[6] share responsibility for the regulation of foods within Canada. In general, the division of powers has the Food Directorate overseeing the establishment of policies and guidelines and CFIA focusing on enforcement and inspection duties related to the food supply. Food products generally do not require premarket approval. Certain nutraceuticals, however, may be considered novel foods as described in the

Food and Drug Regulations.[4] In Canada, novel foods are foods that have been produced through new processes, that do not have a history of safe use as a food, or that have been modified by genetic manipulation.[7] These foods must undergo a safety assessment by the Food Directorate before they can be sold in Canada.[7] Guidelines to assist manufacturers in making novel food submission are published on the Health Canada website.[8] Each submission is considered on a case-by-case basis and the safety assessment is flexible, allowing several different types of evidence to be considered in the review. The submission ultimately must demonstrate that the novel food is safe for consumption and should refrain from jeopardizing the nutritional status of Canadians.[7]

2.2.2 Regulation of Nutraceuticals Considered as Food Products with Health Claims

As noted above, nutraceuticals making therapeutic claims would be considered as drugs under the Food and Drugs Act. Typically, such products are subject to the requirements for drugs under the Food and Drugs Act and its Regulations, however, provisions have been included to exempt certain foods from these requirements. The exemption allows for food health claims to be placed in a table following Section B.01.603 in the Food and Drug Regulations through regulatory amendments following review of the claim by Health Canada. There are currently 20 such claims in the table describing each claim that can be made and all conditions for its use.[4] To add additional claims to the table, a health claim submission must be made to Health Canada for its review.[9]

Health Canada has published guidance documents for preparing Health Claim submissions.[10] These submissions must include a characterization of the food, a characterization of the health effect and an evaluation of evidence supporting the claim validity.[10] Health Canada makes it clear that the health claim must be supported by human studies, however non-human studies may also be presented.[10] The type and number of studies required to demonstrate sufficient evidence depends on a variety of factors.[10]

Only products making therapeutic claims are required to undergo this review process and the subsequent regulatory amendment that will allow them on the market. Health Canada does allow foods that make function claims to enter the market without a pre-market approval process, provided that they are truthful and not misleading.[11] These function claims in Canada are analogous to the structure/function claims associated with the dietary supplements in the US and state the beneficial effects a food has on normal functions or biological activities of the body.[11] Manufacturers making such claims must be able to provide evidence supporting the truthfulness and validity of the claim to Health Canada upon request.[11] Although pre-market approval for these claims is voluntary and not required, Health Canada and the CFIA encourage the practice.

Nutraceuticals in the food category may also make general health claims that do not refer to a specific or general health effect, disease, or health condition.[12] These claims are broad claims that promote health through healthy eating and must be in line with the recommendations outlined in Canada's Food Guide.[12] Under these provisions, a product may use the word healthy in the claim provided it meets the recommendations of healthy eating patterns by Eating Well in Canada's Food Guide.[12] No premarket authorization is required when manufacturers make such claims on their products, however they must ensure they meet the requirements set out lest they become the subject of enforcement action.[12]

2.2.3 Regulation of Nutraceuticals Considered as Drugs

Common throughout all definitions of nutraceuticals is the acknowledgement that they provide some beneficial health effects. As noted above, such a statement falls within the realm of health claims under the Canadian regulatory systems. Nutraceutical products that make claims that do not meet the standards set for food claims, would then be subject to the drug regulatory framework within Canada. In general, drug products in Canada must go through a rigorous premarket approval process through the evaluation of a drug submission replete with the requisite pre-clinical and clinical trial information to support the product's safety and efficacy. The vast majority of nutraceuticals under the Canadian regulatory system however, would be classified into a subset of drugs known as Natural Health Products and be exempt from many of these requirements, allowing for a simpler path to market.

2.2.4 Regulation of Nutraceuticals Considered as Natural Health Products

Natural Health Products (NHPs) are defined and subject to the requirements set out in the Natural Health Products Regulations.[13] The majority of nutraceuticals in Canada would be regulated under this category. Natural Health Products include vitamins and minerals, herbal remedies, homeopathic medicines, traditional medicines, probiotics and other products like amino acids and essential fatty acids and are restricted to oral, topical or sublingual routes of administration.[14] These products cannot be prescription products, drugs administered by puncturing the skin or any substance that is regulated under the Tobacco or the Controlled Drugs and Substances Acts of Canada.[13] Although these products are considered a subset of drugs, they are exempt from many of the requirements in the Food and Drugs Regulations unless specifically referenced in the Natural Health Products Regulations. NHPs are under the authority of the Natural and Non-prescription Health Products Directorate (NNHPD).[14]

The definition of an NHP in the regulations consists of two parts: a function component and a structure component.[13] The function component refers to the product making a drug claim or more specifically the diagnosis, treatment, mitigation or prevention of a disease, disorder or abnormal physical state or its symptoms in humans, restoring or correcting organic functions in humans, modifying organic functions in humans, such as modifying those functions in a manner that maintains or promotes health.[13] The structure component refers to the medicinal ingredient or ingredients in the NHP, and includes ''herbal remedies, traditional and homeopathic medicines and materials derived from plants, algae, bacteria fungi, or non-human animal material, amino acids, essential fatty acids, probiotics, minerals, several vitamins and synthetic duplicates of the natural ingredients''.[13]

2.2.4.1 NHP Licensing Requirements

All manufacturers, packagers, labellers and importers of natural health products are required to hold a site license.[14] The site license will specify exactly which of the previously listed activities the applicant is allowed to carry out with respect to NHPs.[14] In order to obtain a site license, the applicant must demonstrate that they are in compliance with the Good Manufacturing Practices (GMP) requirements set out in the regulations.[14]

NHPs are required to undergo a premarket approval process and obtain a license prior to entering the market.[14] Products receiving approval will be granted a Natural Health Product License and be assigned a Natural Health Product Number.[14] NHP license submissions are reviewed by the NNHPD and applicants must describe the product's ingredients (both medicinal and non-medicinal), its manufacturing process, and proposed claim as well as provide evidence detailing the product's safety, efficacy and quality.[14] The review process is predicated upon a risk–benefit analysis so the amount and type of evidence required will be dependent on a variety of factors.[14] These factors include the product's intended use, dosage, duration of use, target population and the type of claim proposed.[14]

2.2.4.2 NHP Claims

The type of claims made by NHPs can be separated into two categories for classification processes: traditional use claims and modern health claims.[14] Traditional use claims are for products that have been used within a cultural belief system or healing paradigm for at least 50 consecutive years. Unlike other jurisdictions with similar traditional use clauses, the demonstration of the traditional use of the product in a culture or country other than Canada is acceptable; however, the specific cultural paradigm must be referenced in the claim being made. For instance, products making a claim for a product used in Traditional Chinese Medicine are required to include in the product's claim ''Used in Traditional Chinese Medicine for...'' or other similar

wording to clearly indicate the relationship the claim has with the paradigm.[15] Products making traditional use claims are supported by evidence demonstrating their safe and effective use in the traditional paradigm.[15] Traditional claims for products containing more than one medicinal ingredient are allowed provided all such ingredients have evidence showing traditional use for the claim within a single traditional paradigm and the use of these multiple ingredients follows logically from the theories, belief systems and/or experiences specific to the that paradigm.[15]

Modern health claims do not meet the requirements of traditional use claims.[16] These claims can use evidence demonstrating traditional use but will require additional sources of scientific evidence including human studies.[16] Different types of claims would require different types of evidence with higher-level claims requiring a higher level of evidence as support.[16] In order to facilitate the license application, Health Canada has provided several resources including guidance documents,[15,16] which provide information on the types and amount of evidence that is required in an application, and product and ingredient monographs.[17] The product and ingredient monographs can be used as evidence to demonstrate the safety and efficacy of a product or specific ingredients within the product.[17] These monographs provide specific details on the medicinal ingredients intended use, *i.e.* the therapeutic claim that can be made with it, as well as its conditions of use, acceptable sources and forms, dosage information and any risk information associated with it.[17]

License applications for products that conform to all the criteria and conditions set out in a monograph can undergo an expedited compendia application with the monograph being permitted as the sole source of evidence and safety for the product. Such an application would still need to demonstrate the quality of the product through its descriptions of its manufacturing process and its quality summary report.[15,16]

2.2.4.3 Food-like Natural Health Products

NHPs can be in a variety of dosage forms provided they comply with the two components of the NHP definition.[13] They can be found in typical drug or supplement forms such as capsules, pills, tablets, powders and tinctures as well as more conventional food forms including bars, gums, wafers or beverages. Because of the limited restrictions on product form and the ability to make claims, manufacturers have seen the NHP Regulations as a mechanism through which they can add claims to their food products. This was never the intent of the regulations and posed a significant regulatory challenge for the NNHPD.[18] Health Canada took steps to address these ''food-like NHPs'' and developed guidance to facilitate the classification of these products.[18] The decision on classification was based on four criteria: Product Composition, Product Representation, Product Format and Public Perception and History of Use. New products that are submitted as NHPs that are classified as foods would be directed to the food directorate.[18] Any

health claims the product manufacturer would want to make would need to be reviewed under the provisions of the Food and Drugs Act and regulations regarding health claims in food described above.[9]

Health Canada has also been actively reviewing previously authorized NHPs that fall on the food–NHP interface and determining if such products should be reclassified as foods.[18] For those products that are considered foods, Health Canada works with the manufacturers to transition these products to the food regulatory framework. This transition is facilitated through the issuance of Temporary Marketing Authorization Letters by the Food Directorate.[19] In these cases, any therapeutic claims made by the product would no longer be allowed until they are reassessed and approved under the health claims in food provisions described above. A list of all foods that have received temporary marketing authorization letters are can be found on the Health Canada website.[20]

2.2.5 Self-care Products Initiative

Health Canada is currently reviewing its approach to regulating self-care products with the goal of putting into place a regulatory system that will harmonize the regulation of these products.[21] As nutraceuticals fall under the definition of self-care products, changes in the regulation of these products is anticipated. Through the self-care products regulatory initiative, Health Canada has already held several public consultations seeking input on modernizing the department's approach to regulation of these products.[22] In particular Health Canada is looking at the evidence standards required for health claims and extending its risk-based regulatory oversight.[22] The results of these consultations and details of the proposed regulatory framework are expected to be announced in the spring of 2019.[23]

2.3 Overview of Nutraceuticals Regulation in the United States

Under the United States regulatory framework the vast majority of products defined as nutraceuticals are currently regulated as dietary supplements. Over 50% of Americans take some form of dietary supplement every day,[24] and the US dietary supplement industry is a big business with gross sales of over $46 billion in 2018.[25] Contrary to popular belief, dietary supplements are regulated in the United States, although there is controversy about the adequacy of existing regulations.[26] A number of government entities, including states, have jurisdiction over supplements depending on how the products are marketed and what they contain (*e.g.* botanical dietary supplements). A complete overview of these responsibilities is beyond the scope of this chapter and individual state regulations will not be discussed. A few snapshots of national authorities are appropriate in order to provide some insight into the potential complexity of supplement regulations.

The Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) is responsible for keeping crop pathogens and invasive weeds from entering the US in imported produce and other raw materials. For example in the late 1990s, APHIS considered a ban on imported lemongrass *Cymbopogon citratus* (DC) Stapf. (*Poaceae*) because the plant was suspected of harboring a crop pathogen capable of infecting cereal crops. The proposal was withdrawn when APHIS determined that the rust pathogen was already in the US.

The Bureau of Alcohol, Tobacco, Firearms, and Explosives is responsible for collecting revenue from alcoholic beverage sales in the US and has in the past taken a close look at herbal tinctures with a high ethanol content. The Drug Enforcement Administration (DEA) is responsible for enforcing laws surrounding controlled substances, including botanicals such as marijuana (*Cannabis sativa* L.) and khat (*Catha edulis* (Vahl) Forssk. ex Endl.). The US Fish and Wildlife Service enforces the convention on international trade in endangered species (CITES), while the Federal Trade Commission (FTC) is responsible for enforcing truth in advertising.

2.3.1 Food, Drug, and Cosmetic Act (FDCA) and the US Food and Drug Administration

The US Food and Drug Administration (FDA) is the major regulatory agency with responsibility for dietary supplements in the USA. The enabling legislation is the Federal Food, Drug, and Cosmetic Act of 1906 (FDCA), as amended (United States Code Title 21).[27] The agency regulates foods, drugs, cosmetics, medical devices and their manufacture in interstate commerce (including imports). The FDA does not regulate the practice of medicine.

The FDCA provides definitions for each class of regulated good and classifies them based on ''intended use''. Intended use is ascertained by accompanying label claims, advertising materials, or oral or written statements about the product, and once intended use is established, the regulations for that category are applied by the FDA.[27] As will be discussed below, there is no regulatory category for functional food in the US, although certain health claims beyond basic nutrition are permitted on conventional foods and dietary supplements.

Relevant definitions for the major categories of product are: ''...food means (1) articles used for food or drink for man or other animals, (2) chewing gum, and (3) articles used for components of any such article''; and ''...drug means (A) articles recognized in the official United States Pharmacopoeia, official Homoeopathic Pharmacopoeia of the United States, or official National Formulary, or any supplement to any of them; and (B) articles intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease in man or other animals; and (C) articles (other than food) intended to affect the structure or any function of the body of man or other animals; and (D) articles intended for use as a component of any

article specified in clause (A), (B), or (C).''[27] The rest of this chapter is devoted to dietary supplements, but it is necessary to point out that differences between pre-market approval requirements for different classes of regulated goods are often attributable to differences in the philosophy behind the regulations. The philosophy behind food regulation is that with very few exceptions, food is presumed safe and the regulatory framework is focused on keeping the food from being rendered unsafe during harvest, storage, processing, packaging, transport and sale. The philosophy behind drug regulation is that a new drug is presumed unsafe until it has been proven to be generally recognized as safe and effective through scientific procedures.[28] Those procedures and their outcomes are subject to review by the FDA in a premarket review process.[27]

2.3.1.1 Nutrition Labeling and Education Act (NLEA)

The concept that food and nutrients might provide benefits to health beyond basic nutrition is an old one. Starting in 1962, the FDA attempted to revise food regulations to adopt the Recommended Daily Allowance (RDA) and to restrict the amount of each vitamin in products to 150% of the US RDA. The Agency also moved to restrict vitamin combinations that could be sold as dietary supplements. FDA promulgated these regulations in 1973. This move generated intense controversy and Congress quickly overturned the regulations. In 1976, Congress passed the Proxmire Amendments, which became section 411 of the Federal Food, Drug, and Cosmetic Act. They prohibited the FDA from establishing standards to limit the potency of vitamins in food supplements or regulating them as drugs based solely on their potency.[29]

A subsequent flurry of activity began in 1990 with the passage of the Nutrition Labeling and Education Act (NLEA) mandating nutrition labeling on most packaged foods and providing for nutrient content claims and health claims on food labels.[30] As noted, the FDA classifies marketed products depending on their intended use. Prior to the NLEA, foods that listed calorie or sodium content on the label were construed as 'foods for special dietary use,' which was a regulatory category requiring pre-market approval.

Additionally, food labels that made risk reduction claims such as 'heart healthy' were treated as drugs requiring FDA approval. The NLEA responded to consumer demands for mandatory nutrition labeling and healthful food designations. Food labels are now required to contain certain types of information on a principle display panel (PDP), including a statement of identity or name of the food, the net quantity or amount of product, and a standard of identity for standardized food. Labels are also required to have an information panel that provides the name and address of place of business of the manufacturer, an ingredient list, nutrition labeling, required allergy labeling, and label identification of artificial flavoring, coloring, and chemical preservatives. Nutrition labeling is provided in a Nutrition Facts

panel and nutrient content claims may be made in the information panel. In addition to the actual amount of nutrients, the percent daily value/serving must be provided in the Nutrition Facts panel. When it passed the NLEA in 1990, Congress deferred action on dietary supplements.

In May of 2016, the FDA published final rules on a new Nutrition Facts label for packaged foods to reflect updated scientific information, including the link between diet and chronic diseases such as obesity and heart disease.[31] The label is intended to make it easier for consumers to make better informed food choices by providing information on serving size, calories per serving, added sugars, vitamin and mineral content, fat, sodium, cholesterol, carbohydrates, and protein. The original and updated NLEA also defined certain labeling terms such as ''good source'', ''high'', ''more'', ''high potency'', ''light'', and permitted health claims on food.

2.3.1.2 Food Allergen Labeling and Consumer Protection Act

To help consumers avoid the serious health risks posed by food allergens, the US Congress passed the Food Allergen Labeling and Consumer Protection Act of 2004.[32] This Act applies to the labeling of foods regulated by the FDA which includes all dietary supplements and all other foods except poultry, most meats, certain egg products, and most alcoholic beverages. The Act requires that food labels must clearly identify the food source names of any ingredients that are one of the major food allergens or contain any protein derived from a major food allergen.

2.3.1.3 Premarket Requirements for Food

As noted, foods are safe by definition and for the most part do not face premarket scrutiny for safety. Exceptions include food additives, pesticide chemicals, novel foods, irradiated food, and genetically modified food. Health claims are subject to pre-market scrutiny by the FDA. The definition(s) of adulterated food are provided in 21 CFR Section 342 (a) (Poisonous, insanitary, *etc.*, ingredients) through (i) (Noncompliance with sanitary transportation practices).[27] Companies are prohibited from introducing adulterated food into interstate commerce.

2.3.1.4 Health Claims

Health claims describe the relationship between a substance and a disease or health-related condition and relate nutrients to disease/health conditions. Health claims may be made for conventional foods or dietary supplements and must relate nutrient content to disease/health conditions. Health claims require prior authorization by the FDA and there are two types of claim. Unqualified health claims are permitted if there is significant scientific agreement regarding the relationship between the food and a health outcome.[33] A sponsor of the proposed claim must petition the FDA and if it

agrees that the association is supported by evidence provided by the petitioner, the FDA writes a regulation to permit the claim. A list of approved (unqualified) health claims is provided on the FDA website.[33]

Qualified health claims must also be supported by scientific evidence and are made when the evidence does not meet the more rigorous ''significant scientific agreement'' standard.[33] A disclaimer or other qualifying language must be part of the claim in order to accurately communicate to consumers the level of scientific evidence supporting the claim. The FDA does not approve qualified health claim petitions. Instead, food manufacturers petition the agency to consider exercising enforcement discretion for the use of a qualified health claim and if the evidence submitted, claim, and disclaimer are adequate, the FDA issues a Letter of Enforcement Discretion but not a regulation. A number of such letters have been issued by the FDA.[34] A typical claim (claims must contain all of the language provided by the FDA) for the relationship of oleic acid to reduction of cardiovascular disease is: ''Supportive but not conclusive scientific evidence suggests that daily consumption of about $1\frac{1}{2}$ tablespoons (20 g) of oils containing high levels of oleic acid, may reduce the risk of coronary heart disease. To achieve this possible benefit, oleic acid-containing oils should replace fats and oils higher in saturated fat and not increase the total number of calories you eat in a day. One serving of [x] oil provides [x] grams of oleic acid (which is [x] grams of monounsaturated fatty acid).''

2.3.2 Food Safety and Modernization Act

The FDA Food Safety Modernization Act (FSMA)[35] was signed into law by President Obama on January 4, 2011 and enables the FDA to better protect public health by strengthening the food safety system. It contains mandatory preventive and risk-based measures and is intended to replace some parts of older food safety regulations that reacted to outbreaks of food-borne illness rather than preventing them. The law provides the FDA with new enforcement tools and gives the FDA enhanced ability to hold imported foods to the same standards as domestic foods and directs the FDA to build an integrated national food safety system in partnership with state and local authorities. Affected companies and individuals must comply with the requirements for risk-based preventive controls mandated by the FDA Food Safety Modernization Act (FSMA) as well as the modernized Current Good Manufacturing Practices (CGMP, 21 CFR 110) unless an exemption applies.[35] The chief exemption for the purposes of this chapter is that firms required to comply with 21 CFR Part 111 are not required to comply with most of the provisions of the FSMA.[36]

The FSMA was implemented as a series of regulations that became final in September of 2015. The primary outcome requires food facilities to have a food safety plan in place that includes an analysis of hazards and establishment of risk-based preventive controls to minimize or prevent the identified hazards.

The FDA's key new tools are enforcement of mandatory preventive controls for food facilities, mandatory produce safety standards, and authority to prevent intentional contamination. Since preventive controls only work if producers and processors comply with them, the FSMA provides the FDA with new tools for inspection and compliance. These include mandated inspection frequency, records access, and testing by accredited laboratories. The FSMA provided the FDA with new authorities to respond effectively when problems emerge despite preventive controls. These are mandatory recall, expanded administrative detention, suspension of facility registration, enhanced product tracing abilities, and additional recordkeeping for high-risk foods (imports).[3]

Finally, the FSMA gave the FDA new authorities to better ensure that imported products meet US standards and are safe for US consumers. The new authorities include: (1) importer accountability – importers have an explicit responsibility to verify that their foreign suppliers have adequate preventive controls in place to ensure that the food they produce is safe, (2) third party certification – FSMA establishes a program through which qualified third parties can certify that foreign food facilities comply with US food safety standards, (3) certification for high-risk foods – FDA has the authority to require that high-risk imported foods be accompanied by a credible third party certification or other assurance of compliance as a condition of entry into the US, (4) voluntary qualified importer program – FDA must establish a voluntary program for importers that provides for expedited review and entry of foods from participating importers where eligibility is limited to, among other things, importers offering food from certified facilities, and (5) authority to deny entry – the FDA can refuse entry into the US of food from a foreign facility if the FDA is denied access by the facility or the country in which the facility is located.[35]

The FSMA directed the FDA to develop a formal system of collaboration with other government agencies, both domestic (*e.g.* State) and foreign. In doing so, the statute explicitly recognizes that all food safety agencies need to work together in an integrated way to achieve our public health goals.[35]

2.3.2.1 Preventive Controls

The FSMA mandates a series of affirmative preventive controls for all food facilities. These are modeled after successful Hazard Analysis and Critical Control Point (HACCP) controls that have been in place for certain commodities such as seafood. Food facilities are required to implement a written preventive controls plan. This involves: (1) evaluating the hazards that could affect food safety, (2) specifying what preventive steps, or controls, will be put in place to significantly minimize or prevent the hazards, (3) specifying how the facility will monitor these controls to ensure they are working, (4) maintaining routine records of the monitoring, and (5) specifying what actions the facility will take to correct problems that arise.

The Act directed the FDA to establish science-based, minimum standards for the safe production and harvesting of fruits and vegetables. Those standards must consider naturally occurring hazards, as well as those that may be introduced either unintentionally or intentionally and must address soil amendments (materials added to the soil such as compost), hygiene, packaging, temperature controls, and control for animals in the growing area and water.

A vital new mandate in this era of globalization of the food supply is authority to prevent intentional contamination. The FDA was directed by Congress through the FSMA to issue regulations to protect against the intentional adulteration of food, including the establishment of science-based mitigation strategies to prepare and protect the food supply chain at specific vulnerable points.

2.3.3 Dietary Supplement Health and Education Act (DSHEA)

In response to significant public demand spurred in part by comments by the FDA commissioner shortly after the passage of the NLEA that the agency intended to regulate supplements as drugs, Congress passed the Dietary Supplement Health and Education Act of 1994 (DSHEA).[37] In addition to creating the regulatory category ''Dietary Supplements'' (DS) and classifying them as a special category of food, Congress placed regulatory authority over DS with the US Food and Drug Administration (FDA) and created the Office of Dietary Supplements (ODS) at the US National Institutes of Health (NIH).

The DHSEA had two primary goals: to ensure continued consumer access to a wide variety of dietary supplements and to provide consumers with more information about the intended use of dietary supplements.[37] The Act defined dietary supplements as a product (other than tobacco) intended to supplement the diet that bears or contains one or more of the following dietary ingredients: a vitamin, mineral, amino acid, herb or other botanical; or dietary substance for use to supplement the diet by increasing the total dietary intake; or concentrate, metabolite, constituent, extract, or combination of any ingredient described previously. Further, the act states that these products must be ingested and that they do not represent a conventional food or a sole item of a meal or the diet.[37] In addition to defining dietary supplements and dietary ingredients, the DSHEA established labeling regulations and gave the FDA permission to develop current good manufacturing practice regulations for dietary supplements.[37] There are no mandatory formulation standards, dietary ingredient, or product registries for dietary supplements in the United States.

2.3.3.1 Dietary Supplement Labels

Supplement labels are similar to food labels. They must have a principle display panel and an information panel. The PDP must bear a statement of

identity with the words ''Dietary Supplement'', ''Nutritional Supplement'', or ''XXXX Supplement'' and a net quantity. The information panel must include the name and address of the manufacturer, distributor, or packager along with a telephone number for reporting adverse events. Nutrition information must be provided if necessary, but instead of a nutrition facts panel, DS labels must have a supplement facts panel that includes the amount of each dietary ingredient (the purported bioactive) per serving along with the percent daily value provided by this amount of ingredient. If no Daily Value (DV) has been established for the ingredient, an asterisk may be provided in the DV column with the legend ''no daily value established'' at the bottom of the panel. Below the supplement facts panel, all ingredients must be listed by common name in descending order of predominance. If the ingredient is a 'proprietary blend', the amount of the blend must be listed in the facts box, with the ingredients of the blend listed below in descending order of predominance in the blend.

Both health claims and statements of nutritional support (structure/function claims) are permitted on labels. Health claims must be nutrient related and pre-reviewed by the FDA as noted in Section 2.3.1.4. Statements of nutritional support are defined in the DSHEA as a statement that: ''claims a benefit related to a classical nutrient deficiency disease and discloses the prevalence of such disease in the United States (for example, 'vitamin C and scurvy'); ''describes the role of a nutrient or dietary ingredient intended to affect the structure or function in humans'' (for example, 'calcium builds strong bones'); characterizes the documented mechanism by which a nutrient or dietary ingredient acts to maintain such structure or function (for example, 'fiber maintains bowel regularity,' or 'antioxidants maintain cell integrity'); or ''describes general well-being from consumption of a nutrient or dietary ingredient''.[37]

Unlike health claims, structure function claims are not pre-approved by the FDA. The DSHEA does require that the manufacturer has substantiation that the claim is truthful and not misleading and must submit a notification with the text of the claim to the FDA no later than 30 days after marketing the dietary supplement with the claim. While the Act requires that manufacturers submit the text of the claim to FDA, it does not require that substantiation evidence be submitted to the agency. If a dietary supplement label includes a structure/function claim, it must state in a disclaimer that the FDA has not evaluated the claim. The disclaimer must also state that the dietary supplement product is not intended to ''diagnose, treat, cure or prevent any disease,'' because only a drug can legally make such a claim. For more information about the difference between structure/function claims and disease claims, see 21 CFR 101.93, entitled ''Certain Types of Statements for Dietary Supplements,'' and FDA's January 6, 2000 final rule entitled ''Regulations on Statements Made for Dietary Supplements Concerning the Effect of the Product on the Structure or Function of the Body'' (65 Fed. Reg. 1000). As noted, there is no product registry for US Dietary Supplements, but the US National Institutes of Health maintains a searchable voluntary database of DS

labels that is available at no charge to users. The database contains information on product names, manufacturers, ingredients, and claims and has about 90 000 labels at present, with about 1000 new labels added per month.

2.3.3.2 Premarket Requirements for Dietary Supplements

There are no premarket approval requirements for structure/function claims. In keeping with the regulatory philosophy surrounding food, premarket requirements for dietary supplements are related to safety. A dietary supplement that contains a new dietary ingredient (NDI) is deemed adulterated under section 402(f) of the FDCA unless: ''it contains only dietary ingredients that have been present in the food supply as an article used for food in a form in which the food has not been chemically altered or; there is a history of use or other evidence of safety establishing that the dietary ingredient when used under the conditions recommended or suggested in the labeling of the dietary supplement will reasonably be expected to be safe''.[37] A new dietary ingredient is a dietary ingredient that was not marketed in the United States before October 15, 1994.[37]

At least 75 days before the product that contains the NDI is introduced or delivered for introduction into interstate commerce, the manufacturer or distributor of the NDI or dietary supplement with an NDI ''must provide the FDA with information, including any citation to published articles, which is the basis on which the manufacturer or distributor has concluded that a dietary supplement containing such dietary ingredient will reasonably be expected to be safe''.[37] Approved or qualified health claims require FDA approval prior to marketing, but statements of nutritional support (structure/function claims) do not.

2.3.3.3 Current Good Manufacturing Practice in Manufacturing, Packing, Labeling, or Holding Operations for Dietary Supplements

As noted, the DSHEA gave the FDA the authority to promulgate current good manufacturing practice (CGMP) regulations specific to dietary supplements.[36] The final regulation was published in 2007, with full compliance required by all size businesses by 2010 (21 CFR Part 111). Any facility involved with the manufacturing, processing, packaging, or holding of dietary supplements is required to comply with the requirements of 21 CFR Part 111.[36]

The CGMP regulations are intended to assure the identity, purity, composition, and strength of finished supplement products and require manufacturers to set written specifications for the control of every phase of manufacturing, processing, storage, and distribution. Companies must also test to confirm whether specifications are met and must use scientifically valid tests that are suitable for their intended use. Written specifications, test methods, demonstration of suitability and validity of test methods, master manufacturing records and batch records are all required, as is the

collection of adverse event reports. Documentation that processes are followed, that tests are valid and suitable, and that tests against specifications for incoming components and completed finished products have been conducted and that results are acceptable must be kept, and the FDA conducts regular CGMP inspections. Within the boundaries of other parts of the FDCA, such as those dealing with harmful microorganisms, toxic natural constituents, and food additive provisions for components that are not dietary ingredients, manufacturers may set their own specifications, and there are no mandatory product quality requirements such as Pharmacopoeial standards that must be followed. Likewise, there are no mandatory test methods such as those published as AOAC International Official Methods of Analysis[38] or in the Dietary Supplements Compendium of the United States Pharmacopeia.[39]

The DSHEA classifies dietary supplements as food in accordance with that definition in the Federal Food, Drug, and Cosmetic Act.[37] In general, a foreign or domestic facility that manufactures, processes, packs, or holds human food for consumption in the United States has to register with the FDA under section 415 of the FDCA and is subject to the requirements related to preventive controls of the Current Good Manufacturing Practice, Hazard Analysis, and Risk-Based Preventive Controls for Human Food rule, unless subject to an exemption. An exemption for dietary supplements is provided in 21 CFR section 117.5(e) which states that subparts C (hazard analysis and preventive controls requirements) and G (supply-chain program requirements) of 21 CFR part 117 do not apply to any facility with regard to the manufacturing, processing, packaging, or holding of a dietary supplement that is in compliance with the requirements of 21 CFR part 111.[36]

The exemption does not apply to the manufacturing, processing, packing, or holding of dietary ingredients. Dietary ingredients are subject to the requirements of the rule, including the current good manufacturing practice (CGMP) requirements of 21 CFR part 117, subpart B; the hazard analysis and risk based preventive controls requirements of 21 CFR part 117, subpart C; the supply chain program requirements of 21 CFR part 117, subpart G; and the recordkeeping requirements of 21 CFR part 117, subpart F.[37]

2.3.3.4 *Dietary Supplement and Non-prescription Drug Consumer Protection Act*

Reports of severe adverse events associated with the use of dietary supplements have raised concerns about the safety of some ingredients and products. In response to concerns about safety, the FDA created MedWatch, a voluntary system for the reporting of adverse events for consumer products over which it has authority.[40] According to the FDA's definition, an adverse event is an incident of illness or injury that is associated with a product or ingredient. The FDA's adverse event reporting system is intended to: (i) detect adverse events, (ii) generate signals of possible public health concerns,

(iii) assess those signals, and (iv) implement appropriate safety measures.[40] This system provides an essential monitoring tool for identifying potential serious public health issues that may be associated with the use of a particular supplement product.

Recognizing that the MedWatch system is voluntary and that voluntary adverse event reports were relatively rare and often devoid of useful information for follow up, the US Congress created a mandatory reporting requirement for serious adverse event reporting (SAE) for DS and non-prescription drugs in 2006.[41] The law requires that manufacturers provide a telephone number for reporting SAE on product labels and that manufacturers keep SAE records and report them to FDA on a regular basis.

2.3.4 Botanical Drugs

The final category of natural therapeutic goods regulated by the FDA in the United States is the botanical drug. If the product is intended to cure, treat, mitigate, or prevent a disease, it may still be marketed in the US, but it will be regulated as a prescription or non-prescription drug. The FDA provides guidance for Complementary and Alternative Medical practice and products as well as for the approval process for conventional drugs derived from botanicals.[42] The FDA recognizes that botanical drugs are inherently more complex than single chemical entity synthetic new drugs, that the active constituents are more likely to be components in mixtures and that active constituents and their activity are not always well defined. For this reason, some ordinarily mandated pre-clinical studies for products already on the market as DS may be bypassed in early stage research, to be completed at later stages in the approval process. In the end, botanical drugs must undergo the same rigorous pre-market scrutiny for safety and efficacy required of all other drugs.

2.4 Conclusion

As nutraceuticals are not specifically defined in either the Canadian or United States regulatory framework, the ultimate regulatory fate of such a product is very much dependent on what regulatory classification it receives. This situation is not unique to North America with jurisdictions around the world also lacking an overarching regulatory framework specifically designed to encompass the entire umbrella of nutraceutical products. Given the myriad variables that ultimately are used to classify a product, it is not surprising that two similar nutraceutical products could fall into completely separate regulatory frameworks within the same country, let alone across borders. Until a clear regulatory definition for nutraceuticals is established, producers, manufacturers and consumers must be aware that not all nutraceutical products are held to the same regulatory standards.

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CHAPTER 3

Effects of Growing Conditions on Plant Medicinal Bioactives

JILLIAN A. FORSYTH AND SUSAN J. MURCH*

Chemistry, University of British Columbia, Kelowna, British Columbia, Canada, V1V 1V7
*Email: susan.murch@ubc.ca

3.1 Plant Chemistry is the Source of Nutraceuticals

Nutraceuticals are commonly defined as food-derived chemicals that can affect human health. Nutraceuticals and all other foods can be complex assemblies of plant chemicals. Food is either produced directly from a plant, from an organism that grew on a plant or from an animal that fed on plants. Plants cannot travel to search for food, run away from danger or move to a new neighborhood when their current location becomes undesirable, so the only way that plants can interact with their environments is through the production of chemicals.[1] Metabolomics is the detection, identification and quantification of all the chemicals in any biological sample.[2,3] At least 114 000 compounds have been identified in the human metabolome including: peptides, lipids, amino acids, nucleic acids, carbohydrates, organic acids, biogenic amines, vitamins, minerals, food additives, drugs, cosmetics, contaminants, pollutants, and other chemicals that humans ingest or are exposed to in the environment.[4] Somewhere between 5000 and 6000 natural chemicals are produced through human metabolism but the rest come from plants. Each piece of a plant contains 30 000–55 000 distinct chemicals.[3,5] An average cup of coffee has about 8400 distinct chemicals while a cranberry has about 28 000 and a glass of wine has about 8600 chemicals.[3,6–8] For food

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plants, between 700 and 1100 phytochemicals have been fully characterized and we estimate that an average person's diet contains at least 200 000 unknown plant chemicals per day. Some of these chemicals are beneficial to health such as antioxidants, antimicrobials, pre- and pro-biotics, immune stimulators, anxiolytics, adaptogens and so on. Other naturally occurring plant chemicals are neurotoxins, hepatotoxins, carcinogens and other poisons.[9–11]

3.2 Plant Mechanisms for Perceiving the Environment

In order for a plant to survive, it must perceive threats, develop a strategy and reallocate resources to respond[12,13] (Figure 3.1). Plant growth regulators (PGRs) are the primary metabolic signals that perceive changes in the environment and induce plant responses[12,14–18] (Figure 3.1). There are five main classes of PGRs that control the production and distribution of plant chemicals: (1) auxins, such as indole-3-acetic acid (IAA), are involved in cell elongation, the initiation of new roots, root growth, branching and bending of plant tissues toward light, (2) cytokinins, such as benzylaminopurine (BA) and kinetin (KIN), that control plant cell division and the growth of new shoots, and play major roles in seed germination, tissue senescence and maintenance of circadian rhythms, (3) gibberellins (GA) which are involved in regulating the distance between leaves on a stem, the sex of flowers and seed germination, (4) stress hormones such as abscisic acid (ABA), jasmonic acid (JA), brassinosteroids, and salicylic acid (SA) that accumulate to allow plant cells and tissues to respond to drought, insect attacks, fungal and bacterial pathogens and other dangers, and (5) communication hormones such as ethylene (EtOH), which is released by plants in response to stress or wounding to warn other plants of the danger, and karrikins (KAR), which are released by burning plants and trees to induce seeds to germinate for ecosystem recovery after wildfires.[12,19–21] Of these, auxins and cytokinins are the most well studied with differing concentrations controlling cell elongation, cell division, shoot and root initiation and growth, and other metabolic processes. However, newer findings suggest that this traditional view of PGRs may be incomplete as there is significant cross-talk between the PGRs and other classes of compounds such as ascorbic acid, flavonoids, polyamines and brassinosteroids that regulate plant growth.

3.3 Climate Conditions Affect Medicinal Phytochemistry

From the plants' perspective, the metabolites that are bioactive in human health are classified as secondary metabolites in the lifecycle of the plant. By definition, secondary metabolites are low molecular weight molecules that are not required for the primary functions of growth and reproduction but

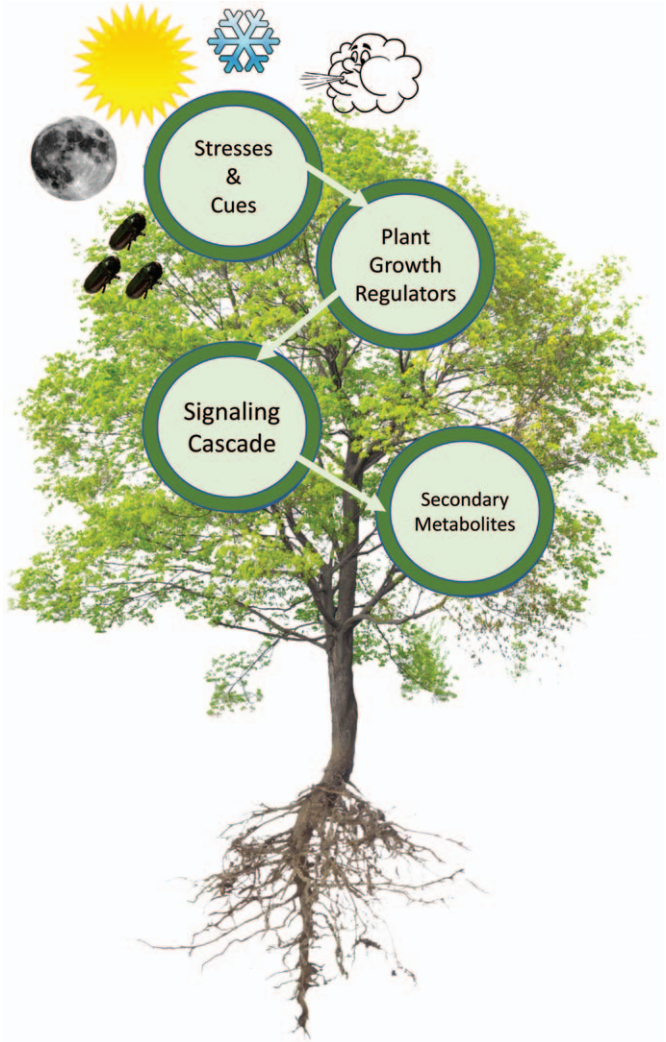

Figure 3.1 Bioactive plant secondary metabolites are produced in response to environmental stimuli *via* a plant growth regulator cascade.

enable plants to adapt and thrive in changing environments.[12,22–24] PGRs act as stimuli to the plant and induce signaling cascades to induce responses in the form of secondary metabolite production, many of which have medicinal functions, are active ingredients in medicinal foods or are sold as nutraceuticals (Figure 3.1).

3.4 Melatonin and Serotonin in Nutraceuticals

Melatonin (MEL) and serotonin (5HT) are neurologically active indoleamines in mammals and produced by many species of plants[16,17]

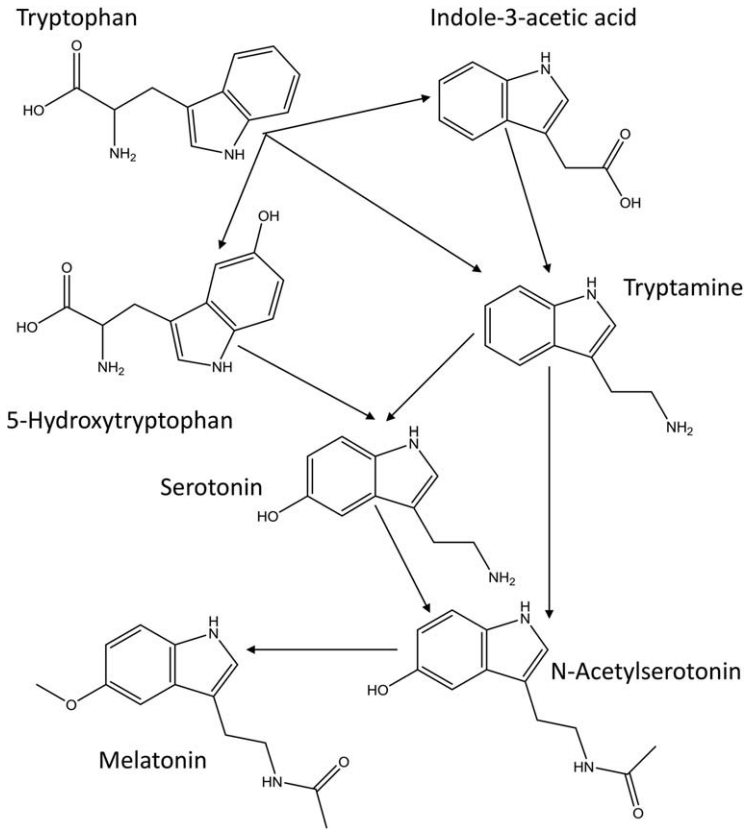

Figure 3.2 Pathway for synthesis of melatonin and serotonin in plants and animals.

(Figure 3.2). MEL has been identified in almost every plant family and in many of the plants we consume as a normal part of a daily diet.[25] Some examples of foods or products that contain significant quantities of MEL include onions, garlic, asparagus, pineapple, banana, rice, ginger, cherries, grapes, walnuts, cranberries, wine, coffee, tea, beer, medicinal plants and herbal supplements.[6,8,24–28] It is possible that MEL and 5HT in the food and supplements we eat may be affecting our health, with direct impacts on circadian rhythms, insomnia, jet lag, anxiety and psychosis as well as recently hypothesized roles of dietary MEL in complex diseases such as cancer, cardiovascular disease and neurodegenerative disease. In a medicinal plant study, Watanabe *et al.* administered traditional Kampo medicinal plant preparations to treat patients with urology pain or non-insulin dependent diabetes.[29] The four different medicinal plant treatments were each complex mixtures of multiple plant species providing a dose of 304, 4320, 5880, or 19 500 pg of MEL per day. The preparation containing the highest amount of MEL contains the root of the medicinal plant *Scutellaria baicalensis*.[29] The concentration of MEL in the plasma was increased by consumption of the

high-MEL medicines and patients with high plasma MEL concentrations complained of sleepiness after a meal, stiff shoulders and nervousness.[29] Patients with low plasma MEL complained that their hands were cold.[29] Several of the medicinal plants and MEL supplements supply higher doses of MEL in a single capsule or tablet.[24] Recently, plant-based MEL supplements consisting of concentrated extracts of cranberry and wine grapes have found their way into European and North American markets. In the USA and Canada, MEL tablets are sold as non-prescription supplements but in the EU, MEL is sold as a prescription drug approved for medical treatment of primary insomnia in people over 55 years of age. The quality of the supplements sold in the USA and Canada can be quite variable with the actual MEL content of tablets found to range from −83% to +478% of the label claim.[30] In one study, many tablets contained the amount of melatonin reported on the labels but some formulations had up to 478% of the amount expected and 8 of the 31 supplement tablets tested contained 5HT in addition to MEL.[30] The European preparations of MEL are of higher quality and are sold as 2–5 mg prescription tablets. The potential for exposure to an unknown and fluctuating dose of MEL through food and drink may provide new understandings of complex nutritional studies and chronic health conditions. Sae-Teaw *et al.* fed a MEL-rich diet of pineapple, orange and bananas to 12 healthy male volunteers and determined that the serum concentration of MEL and antioxidant potential of the serum were both increased by the tropical fruit diet.[31] A similar study of 98 healthy women between the ages of 24 and 55 determined that consumption of a diet rich in high-MEL vegetables increased circulatory MEL and the excretion of MEL metabolites in first morning urine.[32] It has been hypothesized that the Mediterranean diet, which is high in MEL and other plant chemicals such as carotenoids, polyphenols and glucosinolates may combine for health benefits.[33] The Mediterranean diet contains many different MEL sources such as grapes and wine, olive oil, black olives, whole grains, fruits, vegetables, nuts and animal products.[33]

3.5 Melatonin and Serotonin in Plants

The roles of MEL and 5HT in plants are very interesting because it seems that the molecules are both primary and secondary metabolites. Selections of individual seeds, plant breeding programs, chemical mutagenesis and cultures of haploid cells from anthers can all be used to select plant germplasms that produce high concentrations of MEL and 5HT.[34] As primary metabolites, MEL and 5HT have various roles in plant growth and development[17,35,36] and responses to changing climates.[12,14,18]

MEL and 5HT were first identified in plants used in the treatment of neurological illness such as St. John's wort (SJW; *Hypericum perforatum* L.) and Huang-Qin (HQ; *Scutellaria baicalensis* Georg.).[24] Biosynthesis of MEL and 5HT has been fully described in SJW and other medicinal plants (Figure 3.2).[12,15,28] MEL and 5HT are derived from tryptophan in a pathway

that interacts with auxin (indole-3-acetic acid), 5-hydroxytrypophan, tryptamine and dimethyltryptamine in some species.[27,37] The amount of MEL and 5HT in SJW, HQ and other plants depends on the growing conditions, the light levels, the temperature, mineral or metal contamination in soils, insect herbivory and other environmental factors.[12,18,22,27,38,39]

As secondary metabolites, MEL and 5HT may be produced as antioxidants to detoxify reactive oxygen in plant tissues or as anti-herbivory compounds to deter insect feeding.[12,40] 5HT is important for gut function in insects and plant-based 5HT can directly impact the ability of insects to manage bodily fluids.[41,42] The amount of MEL and 5HT in plant-based nutraceutical products depends on the environment under which the plants were grown. Studies have found that MEL concentrations were highest in SJW tissues grown in the dark.[12,43] The rate of metabolism of MEL and 5HT was determined using ^{14}C-labeled tryptophan as the precursor, which revealed that under the dim ambient light of a standard fume hood, the rate of 5HT production was nine times higher than in SJW plants grown under standard, full-spectrum plant growth lights.[28] In SJW grown under daylight growth bulbs, the rates of biosynthesis of MEL was 38 times higher than in plants grown in light.[28] These data were the first indication that the relative ratios of MEL and 5HT may be the mechanism by which a plant detects day and night. In a longer-term study using licorice (*Glycyrrhiza uralensis* (Fisher.)), Afreen *et al.* found that MEL was highest in shoots grown under red light and in roots of plants exposed to UVB light.[44] They proposed that MEL may provide plant protection from UV irradiation.[44] Serotonin responses were observed in *Sedum morganianum* E. Walther, in which the concentration of 5HT was decreased by exposure to green or red light for 12 hours.[45] Interestingly, after 24 hours of a green spectrum, the 5HT concentration increased; however, after 7 days of green light, the 5HT level was not different from controls.[45] Finally, Tiryaki and Keles demonstrated that MEL induced germination of *Phacelia tanacetifolia* Benth. seeds in dark conditions, indicating a potential role for MEL in seed physiology.[46]

Exposure of plant tissues to heat and cold stress has also been shown to affect MEL and 5HT metabolism,[18,36] which could affect the composition of plant-based nutraceuticals. When quantum dots bound to MEL and 5HT were used to visualize the distribution patterns and behavior of MEL and 5HT in SJW roots, it was observed that 5HT travels in a polar direction from the crown to the tip of the root and MEL migrates across the root from the vasculature to the epidermal cells at room temperature, however, under heat or cold stress, the normal patterns of distribution are disrupted and the indoleamines flood the tissues.[18] It has been hypothesized that plants produce MEL and 5HT as antioxidants to protect tissues from temperature stress.[26,36] In a study with the model plant *Arabidopsis thaliana,* Bajwa *et al.* demonstrated that treatment with exogenous MEL induced upregulation of genes that enable plant tissues to redirect resources to respond to cold stress.[36]

The amount of MEL and 5HT in nutraceuticals is also dependent on the soil composition where the plants were grown. Abiotic stresses in the environment such as metal contamination and pollutants induce production of more MEL in plant tissues.[38,47] Optimized growing systems were established to produce specific amounts of MEL, 5HT and other bioactives in SJW and HQ in greenhouses.[39,48–51] Growing these plants to yield specific phytochemical amounts requires an advanced understanding of the environmental and biochemical control of bioactive synthesis.

3.6 Optimizing St. John's Wort Medicinal Chemistry

SJW is a common dietary supplement and nutraceutical sold for treatment of mild-to-moderate depression, anxiety, restlessness, insomnia, neuralgia, pain, and inflammation. Oil extracts are used internally to treat inflammatory gastrointestinal conditions, and topically to treat bites, bruises, burns, hemorrhoids, lacerations, muscle pain, sunburn, urticaria, and wounds.[52] Commercial preparations of SJW contain at least 30 medicinal bioactives[53] including; caffeic acids, chlorogenic acid, catechin, epicatechin, isoquercetrin, rutin, hyperoside, hypericin, pseudohypericin, protopseudohypericin, hyperforin, adhyperforin, and degradation products such as furohyperforin[54] (Figures 3.3 and 3.4). Growing plants to produce the tissues required for production of nutraceuticals requires optimization of the environmental conditions to produce the necessary chemicals.[55] Most SJW preparations are standardized to contain not less than 0.04% of hypericin and pseudohypericin and not less than 0.6% of hyperforin.[52] Hypericin and pseudohypericin are sequestered in glands on the surface of leaves and flowers (Figure 3.5a, white arrow) where they are phototoxic to some insects; other insects have evolved elaborate sequestration mechanisms to detoxify the reactive oxygen produced in metabolism of the phototoxic reactive oxygen generating bioactives (Figure 3.5b).[56] Production and degradation of hypericin and pseudohypericin in the tissues is dependent on light, and exposure of either flowers, glands or isolated extracts to a bright white light or full-spectrum light source is required for complete conversion of the proto-forms into the active forms (Figure 3.3).[57,58] Similarly, the concentration of hyperforin in SJW tissues depends on the growing conditions.[48–50,55,57,58] In general, temperature, water deprivation, light intensity and availability of CO_2 have all increased hyperforin. Interestingly, hyperforin is found not only in SJW but also in *Scutellaria baicalensis* Georg. indicating a more widespread occurrence of the loroglucinols.[39] The concentrations of hypericins and hyperforin in SJW tissues is increased by insect feeding and artificial wounding.[59] Therefore, the yield of hypericin, pseudohypericin and hyperforin in SJW flowers and leaves used for nutraceutical production is dependent on the growing conditions, the stage of flower development, the available nutrition, the amount of CO_2 in the atmosphere, the amount and spectrum of light, insect herbivory and other physical factors.[49,57–59]

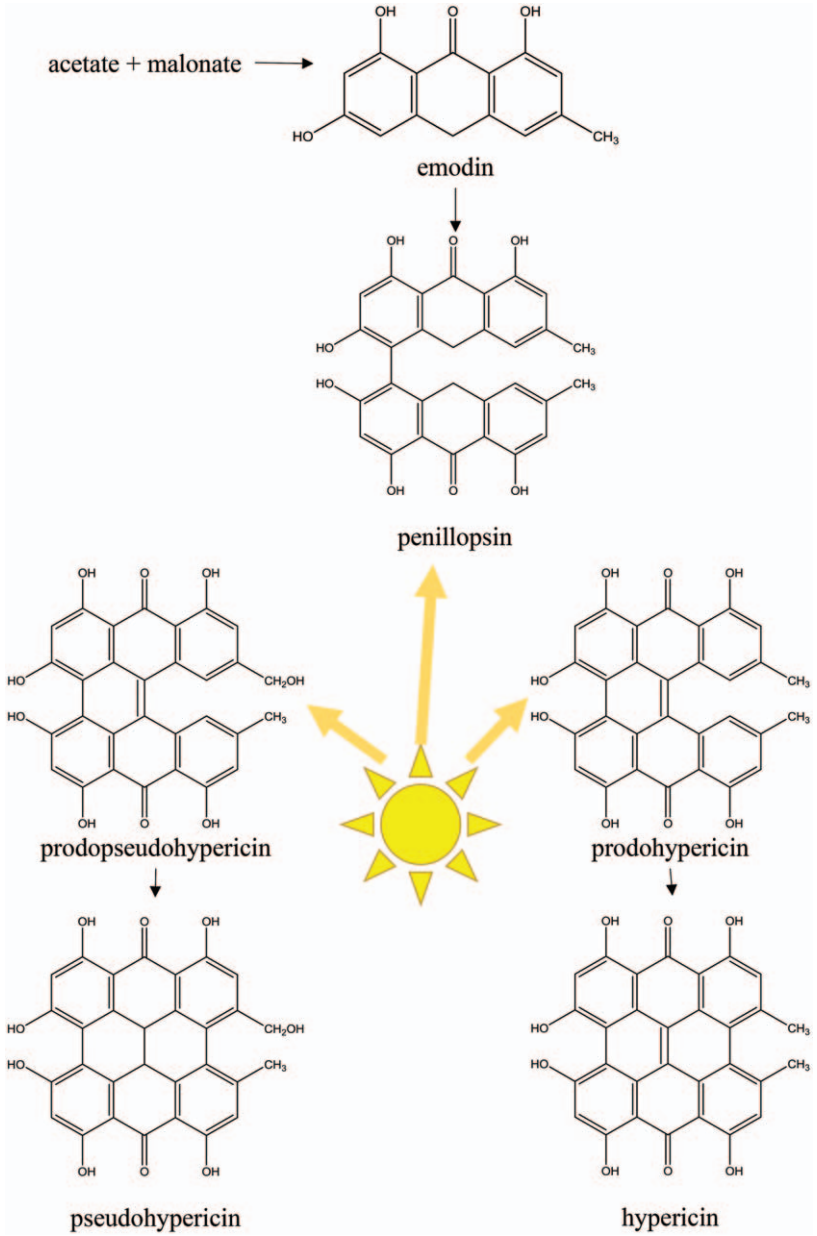

Figure 3.3 Light controls production of hypericin and pseudohypericin from the proto forms.

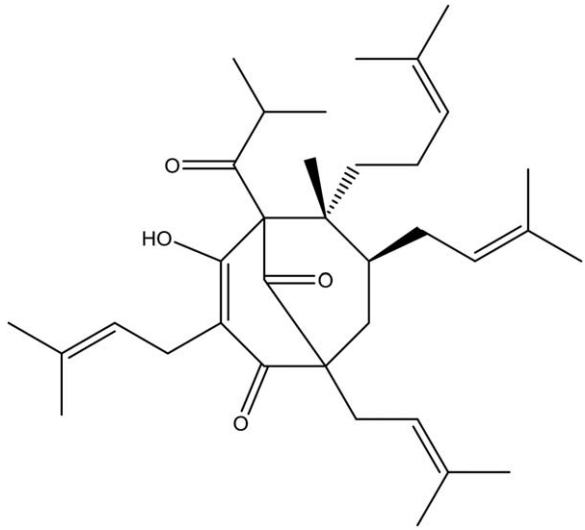

Figure 3.4 Hyperforin is an important insect-feeding deterrent in plants.

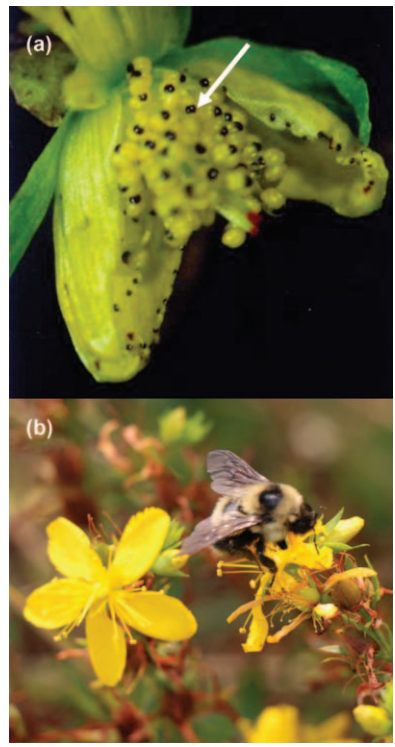

Figure 3.5 Hypericin glands on the surface of St. John's wort (*Hypericum perforatum*) flowers (a; arrow) can be phototoxic to insects that feed on the flowers or attract pollinators (b).

3.7 Conclusions

Plants are complex organisms that respond to their environment by production of chemicals. Most of the plants used in nutraceutical production have not been subjected to the type of breeding programs that are common for food crops and there is huge variability between individual plants. The production of specific phytochemicals requires optimized growing conditions and strict management of the crop environment for optimal yields. In the future, it is likely that medicinal and nutraceutical plants will be grown in sterile controlled environments to ensure high-quality plant material for high-quality products.

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CHAPTER 4

Extraction Technologies for Plant-derived Nutraceuticals and Natural Health Products

CORY S. HARRIS,*[a] TERRY VANDERHEYDEN[b] AND JOHN T. ARNASON[a]

[a] Biology Department 30 Marie Curie, University of Ottawa, K1N 6N5, Canada; [b] St Francis Herb Farm Inc., Combermere ON K0J 1L0, Canada
*Email: charris@uottawa.ca

4.1 Introduction

There are a number of excellent reviews that describe nutraceutical and natural health product (NHP) extraction at an advanced level.[1–3] The purpose of this chapter is to give beginning graduate students and newly hired scientists in industry some insight into appropriate laboratory and industry methods for extraction of different types of bioactive compounds with an emphasis on plant materials and their handling. The vast majority of bioactive molecules in plants are low molecular weight phytochemicals such as phenolics, terpenoids and alkaloids (Figure 4.1). The objectives of extraction are diverse. For research purposes, extraction may be undertaken for discovery of active plant species, or to isolate active biomolecules. These are the basic objectives of drug discovery programs or ethnopharmacology research, which seeks to find the scientific basis of the use of traditional medicines. In industry, extraction may be for preparation and optimization of medicinal dosage forms (tablets, gelcaps *etc.*) to be marketed as NHPs or

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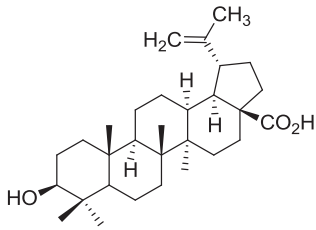
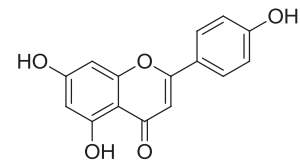
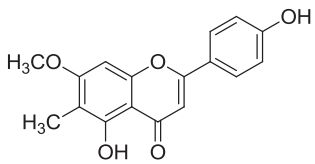


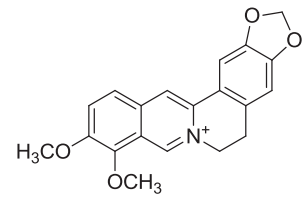
Betulinic acid



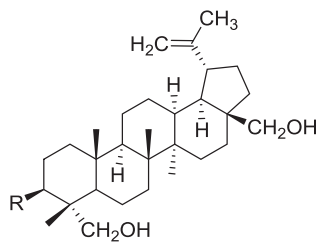
Quercetin



Sideroxylon



Berberine



23,28-dihydroxylupan-20(29)-ene-3β-caffeate,
R = O-caffeoyl

Figure 4.1 Structures of selected bioactive phytochemicals described in the text. Betulinic acid and 23,28-dihydroxylupan-20(29)-ene-3β-caffeate are terpenes, quercetin and sideroxylon are phenolics belonging to the flavonoid grouping and berberine is a β-carboline alkaloid.

for nutraceutical additives in the food industry. In a short review, we have provided a few examples from the literature and tips from our own experience.

4.2 Plant Collection and Identity

These first steps are often where research goes wrong and requires special attention. Even if extracts and preparations are obtained from commercial companies, research on bioactivity, efficacy, safety *etc.* requires that all the issues raised here are fully documented with proof of plant identity and preparation method.

For research purposes, plants may be collected in nature, cultivated or obtained with an ethnobotanical collaborator, or from a wildcrafter or

commercial herbal grower. Permits will be required if collecting in many countries or in parks and public lands. For plants collected with indigenous groups, a collaboration agreement with the community and elders is needed as well as approval from the University research ethics board. Special attention to prior informed consent and intellectual property rights is essential and should be confirmed in formal documents. Because of the biodiversity convention, the permit process in many countries is slow and arduous and should be undertaken well in advance of field work. Local collaborators who have shared intellectual property and co-publication rights can ensure the success of overseas projects. Suggestions for field work are available elsewhere,[4] as are guidelines for ethical research with Indigenous Peoples.[5]

Research papers and registration applications for products are often rejected because the identity of the plant material is in doubt. For example, there are many species of cultivated blueberries and simply stating that the raw material is ''blueberry'' is inadequate. In Eastern North America, *Vaccinium angustifolium* is the widely harvested lowbush blueberry, *Vaccinium corymbosum* is cultivated highbush blueberry, while many more *Vaccinium* species are wild-harvested in North and South America. Each has a unique phytochemical and activity profile,[6] so it is essential to properly characterize the plant material with a representative specimen (called a voucher), with leaf, stem, flower, root if possible, collected and preserved in a plant press.[7] The press should be dried over low heat to prevent mold growth. Heat produced at the back of an electric (not propane) refrigerator or by a light bulb is frequently used by experienced field collectors, as are electric heaters set at low power with a fan. The voucher is then mounted with a herbarium label and submitted to a taxonomist for identification and placed in a herbarium as a ''voucher specimen''. The label should have: scientific name, taxonomic authority and family, herbarium number, date of collection, locality (latitude, longitude, altitude by GPS and country, province, village), habitat (ex. veg type, soil, moisture conditions) name of collector, name of taxonomist, remarks: local name, use and descriptive notes. It is essential to report the voucher number of the specimen in theses, reports and research articles.

Whereas voucher specimens support accurate species identification in academic research, plant identity can be a problem in other situations. Industrial finished-product manufacturers often do not grow or collect their own products. To offer a wide diversity of medicinal preparations, they often purchase bulk dried powdered herbs from national and international supply companies. Here, identity is an important issue. In Asia, species in Traditional Chinese Medicine are often substituted when the desired species is unavailable. Substitution of black cohosh, *Actaea racemosa,* with an Asian *Actaea* sp. was connected to incidents of hepatotoxicity in Canada. Identity of dried ground plants can be assured by an experienced herbalist, phytochemical analysis, or DNA methods *etc.* Adulteration of herbs with pharmaceuticals and substitution of costly commercial products is unfortunately a frequent problem. When a single or a few phytochemical markers are used for quality assurance by high-pressure liquid

chromatography (HPLC), a fraudulent product can remain undetected. Recently metabolomic methods that look at the whole phytochemical profile of a product or sample are in use and more easily detect identity problems.[8] Watch groups such as the American Botanical Council's botanical adulterants prevention program (published in the trade magazine *HerbalGram*), research societies like Natural Health Product Research Society and regulatory agencies pay special attention to these issues.

4.3 Fresh, Fermented, Enzyme Treated or Dried Plant Materials

Nutraceutical food items and herbal NHPs or dietary supplements can be processed fresh or dried. Grinding, blending and processing living plant material may release enzymes that convert stored phytochemicals into a different bioactive form. These include myrosinase, which converts glucosinolates into isothiocyanates and nitriles, and polyphenol oxidase, which converts *ortho*-substituted diphenols into active quinones.

Fermentation has been traditionally used for many years in Asian food and nutraceutical preparations, such as in soya sauce, kimchi, and kombucha, but is gaining popularity in NHP preparations such as fermented ginseng and blueberry. Blueberry fermented with its own skin coat bacterium *Serratia vaccinii* produced a very active product with antidiabetic and other medicinal properties.[9] The bacterium both transforms the blueberry phytochemicals and adds *de novo* compounds to the product.

In addition, the use of enzyme preparations has been introduced as a way of releasing plant bioactives from complex plant matrices. Enzymes such as pectinases, cellulases, and proteases target plant cell walls and have been used to release plant oils such as garlic oils.

The founder of Trout Lake Farm and Klickitat Organics, Lon Ball commented that good commercial drying is the most important step in commercial production of many herbal products, since dried materials are the most practical ones for commercial use. To paraphrase, if the process goes wrong, the herb is worthless. Drying too hot (*i.e.* >60 °C) can destroy many active phytochemicals, which are heat labile. Slow or delayed drying can lead to mold or, worse still, the growth of pathogenic bacteria such as *Escherichia coli* O157 : H7, which is often present in unsterilized organic fertilizer produced with cow manure. An herb drier that circulates warm dry air is an essential piece of equipment for producing dry herbs in a timely fashion. For small-scale field work, a home fruit drier is adequate. Set the temperature at 35–55 °C. For medium scale work (up to 100 kg), an herb drier with plant material dispersed thinly on trays provides a hot-air source and fans to circulate air. It can dry leaf material overnight and roots in a few days. Large-scale commercial operations must dry 1–2 tons per day to keep up with field production and prevent molding. One option developed recently at Amway's Trout Lake Farm is full-sized drying rooms equipped with

a perforated steel floor on which the plant material is placed. Hot air is generated by large-scale propane burners and circulated with powerful industrial fans into a room below the perforated floor. The pressure is sufficient to lift the plant material and dry it in 24 h, producing a very high-quality stable product. Fruits such as berries are often homogenized and dried with a continuous-belt infrared (IR) reflectance system that produces a dry fruit ''leather''.

4.4 Grinding and Sieving

The next step in preparation for extraction is the milling of plant material into a usable form. For most lab applications, a Wiley mill is convenient. The product should be sieved through a screen such as the 30-mesh screen to provide uniform particles for reproducible extraction. Finer milling, for example with a ball mill is used for seed coats or bark, to produce very small size particles. Courser ''tea bag cut'' is occasionally used in industry.

4.5 Extraction Methods

4.5.1 Simple Solvent Extraction of Crude Extracts for Bioassay Screening or Industrial Use

For lab studies to produce an extract for testing in bioassays, the plant material can be extracted in one or a combination of solvents. Whereas water will selectively extract polar compounds (*e.g.* sugars, amino acids), non-polar solvents like ethyl acetate or hexane will selectively extract lipophilic phytochemicals (*e.g.* terpenes, polyacetylenes), while methanol and ethanol provide a more broad spectrum extract. Due to methanol's toxicity, ethanol is usually preferred and can be mixed with water; commercial herbal tinctures, for example, are typically prepared as 55% or 70% ethanol extracts. If little is known about the species' phytochemistry or bioactive molecules, the material can be first extracted in alcohol, which is typical of many commercial preparations, or hot water, which is used in many traditional preparations (decoction or infusion). Usually, the dry plant material and solvent (1 : 20 w : v) is placed in an Erlenmeyer flask and stirred or shaken overnight. For a more thorough extraction, the process can be repeated by filtering the first wash (*e.g.* through a Buchner funnel with Whatman #1 paper), re-extracting the residual plant material with alcohol and then pooling the filtrates. Extraction conditions can be experimentally optimized based on particle size (grinding), solvent-to-material ratio, shaking time, *etc.*, to maximize yield of total extract or specific phytochemicals.

After filtration, the alcohol can be removed with a rotary evaporator (see below) but water (and hydro-alcoholic) extracts require freeze drying – a tedious process – to generate a dry powder. Both methods extract similar phytochemicals, although the alcohol extracts contain higher amounts of the lipophilic compounds and the water extracts may have more hydrophilic

compounds[10] including many polysaccharides that make enzyme and receptor binding assays challenging. Other solvents are used (methanol, hexane, ethylacetate, acetone, *etc.*) but may raise residue issues when the product eventually gets registered.

The US National Cancer Institute has screened 65 000 plant extracts for anticancer activity and they developed a two-stage screening method that produces a lipophilic extract with 1 : 1 dichloromethane and methanol that is then rotary evaporated to dryness.[11] The marc (plant residue from filtration) is re-extracted with water to produce a hydrophilic extract and freeze dried. The yield of extracts should be noted and the extract is redissolved with warming and vortexing in a concentrated stock solution (*e.g.* 10 mg extract mL^{-1} in alcohol or dimethyl sulfoxide (DMSO)) for addition to a bioassay (*e.g.* 5 μl extract mL^{-1}). A vehicle control (addition of solvent without extract) should always be tested and, a rule of thumb is that >1% solvent is unacceptable in most assays, with levels <0.5 or <0.1% preferred for cell-based assays.

4.5.2 Targeted Extraction

When an active group of compounds is targeted, a more tailored approach is possible. For example, ginsenosides from ginseng are very stable at higher temperatures and can be extracted efficiently by 1–2 h refluxing or soxhlet extraction in 80% alcohol, which would not be recommended for other less stable phytochemicals. The soxhlet extraction uses a heater to boil a solvent in a round-bottom flask and the vapor is converted to hot liquid in a condenser that drips through a paper thimble containing the plant material to be extracted. The continuous extraction can be left for several hours or overnight and often can remove >95% of soluble active materials from the plant. Alkaloids are best extracted with fractionation between water, at an appropriate pH, and with an organic solvent.

Pure compounds or highly purified extracts can occasionally be prepared by solvent extraction. For example, alkaloids can be crystalized from plant extracts or cannabinoids can be prepared by salt-precipitation of acids from cannabis extracts. Normally, pure compounds can only be isolated from plant products by sequential fractionation, column chromatography and preparative HPLC methods, as described in Section 4.7.

Simple solvent extraction is used in industry for production of traditional tinctures, which typically involves maceration of 1 part herb material with 5 parts of 20–40% alcohol in water for several weeks. Fluid extracts in alcohol require a more concentrated extract, 1 : 1 (w : v).

4.5.3 Automated Solvent Extraction and Subcritical Water Extractions

Extraction procedures in the lab can be automated with commercial extractors such as the Automated Solvent Extractor (ASE), which provide programmed control over solvent mixtures, temperature (up to 200 °C), pressure

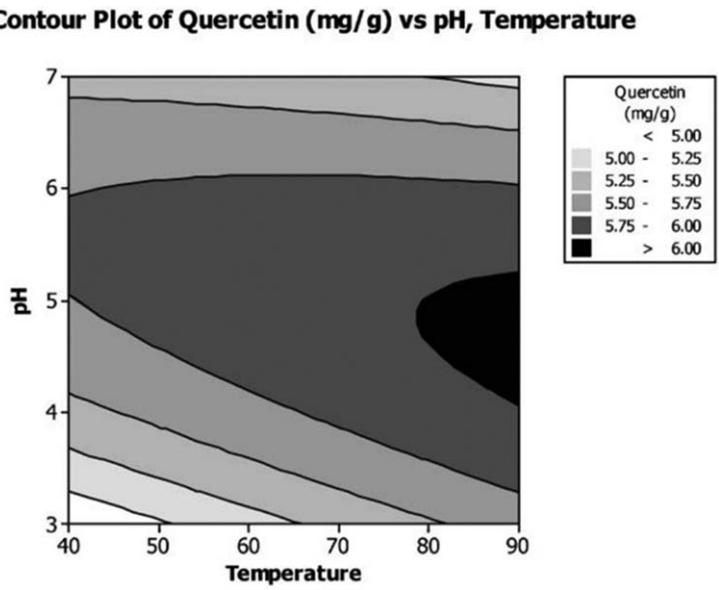


Figure 4.2 Subcritical water extraction of onion for quercetin. Response surface of quercetin yield (mg g^{-1} onion) *versus* pH and temperature of the reaction. Reproduced from ref. 13 with permission from the Royal Society of Chemistry.

(up to 15 MPa) and time of extraction. This equipment increases the flexibility and efficiency of extraction. High pressures and temperatures increase diffusibility of solvent into plant matrices. Typically, organic solvents are used. In extraction of the anxiolytic plant *Souroubea sympetala*, ASE (Figure 4.3) improved extract yield 3-fold compared with conventional ethyl acetate solvent extraction.[12]

When water is used as the solvent at moderately high pressure and temperature the process is called subcritical water extraction.[13] The subcritical conditions increase the dielectric constant of water, which make it very effective for extraction of phenolics in fruits and vegetables. In an example of this method, onion was extracted with pressurized hot water to efficiently extract quercetin glycosides, followed by biocatalytic conversion of the quercetin glycosides into quercetin and carbohydrates. Optimization of the method for temperature and pH is shown (Figure 4.2).

4.5.4 Supercritical Extraction

Supercritical fluid extraction (SCE) using CO_2 at temperatures and pressures where liquid, gas and solid co-exist is ideal for lipophilic compounds while leaving hydrophilic compounds behind. It provides a sterile, solvent-free extract with low risk of oxidation. For example, extraction of St John's wort provided a hyperforin-rich extract for depression without phototoxic

hypericin.[14] Echinacea extracts were enriched in alkylamides without hydrophilic caffeic acid derivatives. Mullally *et al.*[12] found that the anxiolytic extract of *Souroubea sympetala* was more enriched in the active principle betulinic acid by SCE compared with ASE, ultrasonic, soxhlet and ethyl acetate extraction (Figure 4.3). SCE is ideal for removing lipophilic cannabinoids from cannabis and is now a favorite technology in this new industry. However, SCE technology remains relatively slow, potentially hazardous due

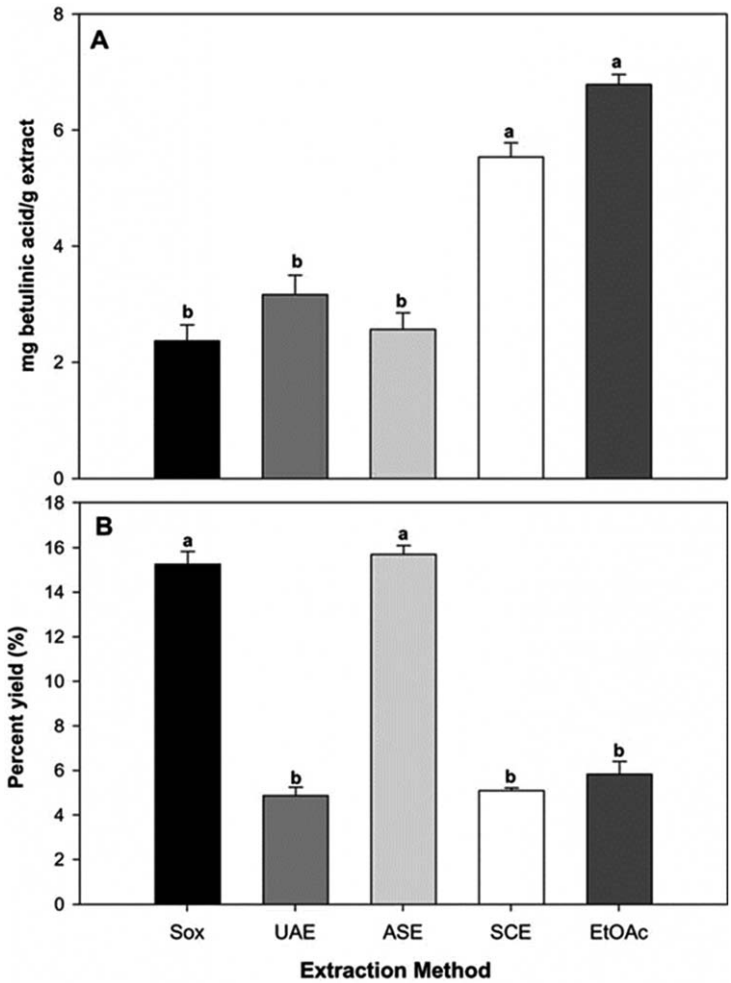


Figure 4.3 Comparison of extraction methods for (A) betulinic acid concentration and (B) crude extract yield from *Souroubea*. Sox = soxhlet, UAE = ultrasonic assisted, ASE = accelerated solvent extraction, SCE = supercritical extraction, EtOAc = ethyl acetate. Bars with the same letter (a, b) are not significantly different ($p = 0.05$).
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to high pressures, and is expensive. Also, the extraction requires optimization for pressure and temperature and cannot extract hydrophilic molecules without a co-solvent (so extracts would no longer be solvent-free). When dealing with plant-based materials, SCE may also extract waxes and structural lipids that can subsequently be removed through additional processing known as winterization (*e.g.* re-extraction with cold ethanol to capture desired metabolites and exclude unwanted components).

4.5.5 Steam Distillation

Essential oils (EOs) are a special low molecular weight product typically prepared by steam distillation (hydrodistillation) of plant materials.[15] The EOs are low molecular weight volatile compounds often sequestered in plant oil glands that can be removed by steam heat and then trapped in a condenser. EOs are composed of many compounds such as mono- and sesquiterpenes, simple phenolics and aliphatic compounds. They are often used as flavoring agents for foods (*e.g.* mint or lemon oil) or as insect repellents/natural insecticides (*e.g.*, eucalyptus or catnip oil) or for aromatherapy. Although EOs are complex mixtures, the component compounds are well studied for toxicity and often have a good safety profile, except for infants.

4.5.6 Cold Finger Extraction

Whole-spectrum extracts that preserve as many of the bioactives in plants as possible can be achieved by cold finger extraction. In this procedure, the plant material is percolated by solvent (usually alcohol) in a reflux system at low temperature. This is achieved by using a partial vacuum and a refrigerated cold finger used to condense the solvent. Extracts prepared by this method have a low risk of thermal degradation, such as oxidation, and preserve low molecular weight aroma compounds and higher molecular weight phytochemical bioactives.

4.5.7 Screw Press and Cold Press

Some technologies used in industry are not routine in lab practice. One of these is the production of plant juices using a screw press. An example is the production of juice products from fresh cut Echinacea herb. The expressed juice is immediately cooled and treated with alcohol to prevent bacterial growth. This juice product preserves high molecular weight polysaccharides and glycoproteins with immunostimulant properties. This product is spray dried and used in immune support products such as Triple Guard Echinacea. Cold press uses high hydraulic pressure to crush fresh material and is routinely used to remove oils from oils seeds, such as canola, sunflower and corn.

4.5.8 Emerging Technologies

Microwave-assisted process (MAP), ohmic heating (OH), pulsed electric field (PEF) or ultrasonic extraction (USE) technologies are emerging technologies used to enhance the release of bioactives from plant matrices.[2] In MAP, fresh plant material is immersed in solvent (usually hexane) and is irradiated with microwave radiation tuned to water absorption. The plant material is heated by the microwave and releases phytochemicals to the solvent. The technique is efficient for extraction of EOs. Similarly, OH uses the fresh plant material as an electrical resistor that heats on application of electrical current. PEF targets cell membranes to release bioactives. Ultrasound is the most accessible technology and a lab ultrasound bath can assist penetration of solvent into plant material and increase yields of phytochemicals.

4.6 Drying of Extracts

An essential and widely available piece of equipment for preparation of dried extracts in the laboratory is the rotary evaporator, which removes solvent form extracts at low temperature. It works by placing the extract in a round-bottom flask, which is rotated in a hot-water bath. The equipment achieves solvent evaporation at a reduced boiling temperature by creating a vacuum in the apparatus with a water aspirator or vacuum pump. The solvent vapor moves to a condenser where it is condensed by a water-cooled coil (or dry ice-filled condenser) made of glass. Solvent then drips into a receiving flask for eventual disposal or reuse. Water extracts are frozen in a round-bottom flask and connected to a lyophilizer (freeze drier), which removes the water by sublimation in a vacuum. The rotary evaporator or lyophilizer often produces glassy or sticky extracts which are challenging to work with. Occasionally, maltodextrin or microcrystalline cellulose may be added as an inert ingredient to produce a powder that is easier to work with. Both water and alcohol extracts can be removed by spray drying, where the extract is forced through a nozzle into a chamber to evaporate solvent. While spray-dried extracts are often desirable powders, the process can lead to oxidation and the equipment is not often available in most phytochemistry labs. When multiple extracts are produced in the lab, a vacuum centrifuge can evaporate multiple extracts quickly and simultaneously but with limited capacity in terms of sample volume (generally <40 mL per sample).

At an industrial scale, spray drying or falling film applications are often used for large-scale extract drying, as these are cost effective and rapid.

4.7 Bioassay Guided Isolation of Active Principles

The identification of active principles is an important research goal that can be achieved by bioassay guided isolation, where an extract is separated into fractions and each fraction is tested for activity.[15] Often, this involves classic manual ''bucket phytochemistry'', although more automated methods are now available. In one classic method, plant material is placed in a Buchner funnel and sequentially extracted with a lipophilic to hydrophilic series of

solvents (hexane, dichloromethane, methanol, water). Each extract is collected and tested in the bioassay for activity, then the most active extract is separated into 10–20 fractions by gradient column chromatography on silica gel or another adsorbent. Usually a solvent mix (*e.g.* hexane; ethyl acetate for silica gel) is added in 50 ml aliquots with an increasing proportion of the polar solvent. The collected fractions are either evaporated and tested for activity or first screened for complexity (*e.g.* thin-layer chromatography, HPLC), pooled based on similarity, and then evaporated and tested for activity. The most active fractions are re-chromatographed and the secondary fractions tested for activity. Active secondary fractions are sent for preparation of pure compound by preparative HPLC. The isolated compound is identified by spectroscopic means (nuclear magnetic resonance (NMR), mass spectroscopy (MS) and infrared spectroscopy (IR)) or X-ray analysis if crystals can be obtained. Usually, ^{13}C and ^{1}H NMR spectra are required with modern correlative spectroscopy (COSY) to provide an unambiguous identification of the structure. An example[16] is shown in Figure 4.4 for the isolation of an antidiabetic triterpene from mountain ash bark. The bioassay

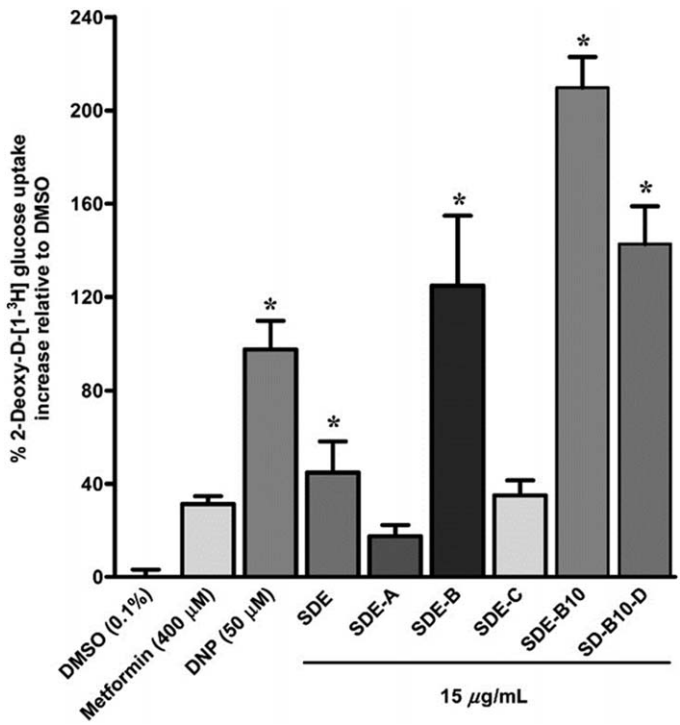

Figure 4.4 Bioassay guided isolation of antidiabetic fractions (SDE, SDE-A, SDE-B, SDE-C, SDE-B10, SD-B10-D) leading to isolation of a single bioactive triterpene from mountain ash. Fractions with a star were active in an assay for glucose uptake by muscle cells. DNP = dinitrophenol.
Reproduced from ref. 16 with permission from American Chemical Society, Copyright 2010.

used was stimulation of glucose uptake in muscle cells. Active fractions are detected by the assay (* in Figure 4.4) and further fractionation occurred until the final pure active substance was obtained.

Today, bioactivity assays are highly varied due to the many innovations in biopharmaceutical research. Typically, *in vitro* multi-well plate assays will be used and allow for an automated evaluation of activity in control, vehicle control, positive control and different concentrations of the crude extract and fractions or pure compounds. For example, a gamma aminobutyric acid (GABA) transaminase enzyme inhibition assay conducted in a microplate was used to identify the calming active principle of lemon balm (rosmarinic acid).[17]

Some recent automated innovations have improved bioassay guided isolations. Now fractions prepared by semipreparative HPLC (eliminating tedious open-column methods) can be adsorbed on solid-phase extraction columns and eluted for bioassay testing and compound identification. Whereas 2–3 mg of a pure compound were once required for structure elucidation, use of cryoprobes in NMR can significantly reduce the amount needed to only 100–200 μg of pure compound.

Special automated techniques are also used for separation of complex mixtures such as alkaloids or saponins, where countercurrent chromatography is the method of choice to separate individual compounds. In this technique, two immiscible solvents run past each other leading to resolved compound separation. Leitao *et al.* used this system to separate two quinolone alkaloids in Brazilian *Choisya ternata*, which were then tested for antioxidant capacity.[18] The alkaloids were identified as anhydroevoxine and chosiyine and inhibited the generation of reactive oxygen species in lipopolysaccharide-stimulated leukocytes.

Alternatively, metabolomics can provide a faster method for identifying activity biomarkers in extracts. In a recent experiment,[19] we compared boreal plant species extracts that were active in a diabetic assay, with species that were inactive. The metabolome of each extract was recorded using ultra-performance liquid chromatography (UPLC) + quadrupole time-of-flight mass spectrometry (QTOF-MS). In this technique, complex extracts were separated into 800+ compounds through a small-bore reverse-phase column at very high pressure. Each compound was sent to the TOF mass spectrometer to characterize the high-resolution mass spectrum and molecular formula for parent and fragmented ions with corresponding retention times. Using discriminant statistical analysis, the metabolome of the active and inactive species was compared. A significant biomarker for activity was discovered in the active plants and identified as quercetin 5-*O*-arabinoside from its high-resolution MS spectrum. The compound was tested in the antidiabetic assay and bioactivity was confirmed (Figure 4.5).

One of the interesting features of nutraceuticals and NHPs is the interaction of different active principles (polypharmacy). Researchers at North Carolina State University[20] developed a bioassay method to identify interacting phytochemicals in plants. Their extraction of the commercial antimicrobial plant, goldenseal, led to the identification of a flavonoid fraction

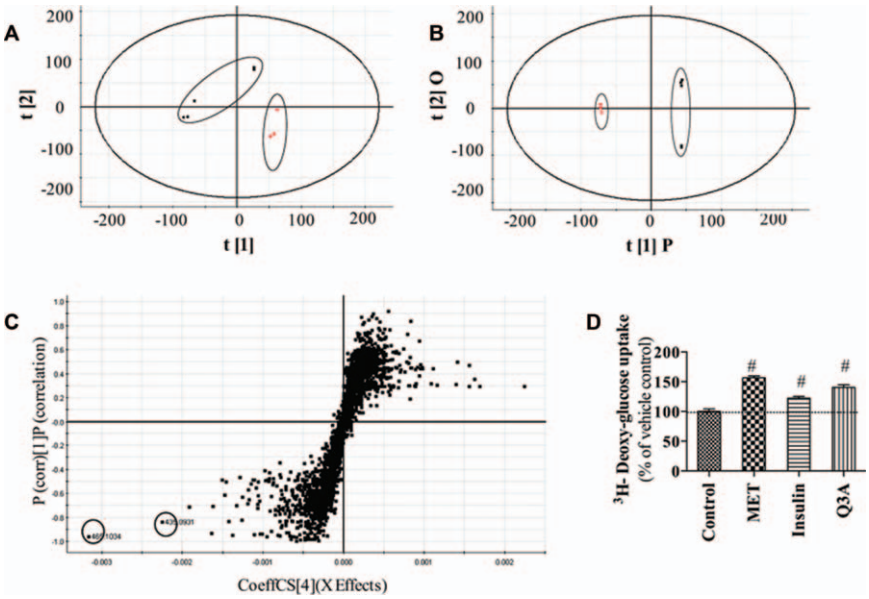

Figure 4.5 Metabolites analysis of selected hot-water extracts based on stimulating glucose-uptake activity. Principal component analysis (PCA) scores (A), orthogonal projections to latent structures discriminant analysis (OPLS-DA) scores (B), S-plot (C) of UPLC-QTOF metabolome of hot water extracts (HWE) of Cree plants. Those stimulating glucose transport (*Rhododenron gromenlandicum, Rhododendron tomentosum*, and *Sarracinea purpurea*) grouped seperately from inactive ones (*Kalmia angustifolia* was found to be an outlier and was excluded from the process). The 95% confidence interval for each group is given. In the S-plot, the metabolome of the active plants was compared with the inactive plants to identify discriminant biomarkers with *K. angustifolia* excluded. In (D), quercetin 3-O-α-L-arabinopyranoside (Q3A, 50 μM) stimulated glucose in C2C12 cells, 140% compared with vehicle control. Metformin (MET) is the positive control. The symbol # on a bar indicates values that are not significantly different ($p = 0.05$).

that enhanced antimicrobial activity of the alkaloids berberine and hydrastine. The mechanism appears to be through inhibition of a bacterial efflux transporter that removes the alkaloids quickly form the bacterium.

4.8 Standardization; Good Laboratory Practices (GLP)

While not required for registration of herbal products in many countries, standardized extracts are a best practice for evidence-based herbal medicines or nutraceuticals. These are reproducible materials tested in clinical trials for efficacy that can be produced by comparable methods for commercial products and therefore will be expected to produce similar results.

Extracts are standardized to active principle(s) concentration ±20%. An example of a standardized product is Asian ginseng (*Panax ginseng* Meyer) extract, G115® Ginseng extract,[21] which contains 4% ginsenosides and has been evaluated in numerous applications. For example, oral administration of G115® in acute and multiple doses is associated with statistically significant improvements in cognitive function.[21]

To prepare standardized extracts it is important to establish Standard Operating Procedures from source plant material (preferably grown under good agriculture practices), using GLP conditions including vouchers, reserve samples, rigorously cleaned equipment and food grade solvents. Active principles are determined usually by HPLC and extracts blended to targeted biomarker concentrations. Standardized, clinically evaluated materials often represent the best-quality products on the market.

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CHAPTER 5

Analytical Approaches for Characterization of Bioactives in Plant-based Natural Health and Food Products

MICHAEL CHAN,[a] JOSEPH M. BETZ,[b] FIONA J. M. TYMM[a] AND PAULA N. BROWN*[a]

[a] Natural Health and Food Products Research Group, British Columbia Institute of Technology, Burnaby, BC, Canada V5G3H2; [b] National Institutes of Health, Office of Dietary Supplements, Bethesda, MD 20892, USA
*Email: paula_brown@bcit.ca

5.1 Introduction

Conventional thought would consider food and medicine as separate entities. The former is ingested to provide nutritional support whereas the latter is ingested (injected, inhaled, or rubbed on the skin) for its physiological effect often to treat, cure, or prevent a disease. Yet, the interface between these two categories is not at all clear or distinct and the market contains products that share attributes of both foods and drugs. The concepts behind products termed medicinal foods, functional foods, and nutraceuticals are not new, as evidenced by the famous quote credited to Hippocrates ''Let food be thy medicine and medicine be thy food''.[1]

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Consumers and regulators demand products meet certain quality and safety standards, regardless of a classification as a food or drug. In the case of products having a therapeutic effect, standards for efficacy would also apply. Such standards are typically assured through the analysis of chemical compounds responsible for the salubrious effects. For conventional foods, proximate analyses are performed with a focus on the evaluation of the macronutrients and micronutrients such as proteins, fats, carbohydrates, vitamins and minerals. Drug testing focuses on analysis of the therapeutic compound or active pharmaceutical ingredient (API), which are typically relatively small synthetic, semi-synthetic molecules or plant secondary metabolites. With functional foods and nutraceuticals that have purported therapeutic effects, both micronutrients (*e.g.* vitamins) and bioactive secondary metabolites are of interest.

The focus of this chapter is to provide a summary of different analytical techniques used to quantify bioactives in functional foods and nutraceuticals. A review of conventional and legislative definitions of foods, drugs and nutraceuticals will proceed an overview of quality testing parameters, namely identity, purity and potency or strength. The chapter includes a discussion of method selection, optimization and validation, and how all these factors tie into the consideration of a method's fitness for purpose. The chapter will conclude with a brief discussion that provides information on documented sources of analytical methods, resources and suggested best practices when characterizing materials for further study.

5.1.1 Foods, Natural Health Products and Nutraceuticals – Regulatory and/or Accepted Definition

In the US, food means ''(1) articles used for food or drink for man or other animals, (2) chewing gum, and (3) articles used for components of any such article''.[2] Similarly in Canada, a food is defined as ''any article manufactured, sold or represented for use as food or drink for human beings, chewing gum and any ingredient that may be mixed with food for any purpose whatever''.[3] The definition of a drug in the US means ''(A) articles recognized in the official United States Pharmacopoeia, official Homoeopathic Pharmacopoeia of the United States, or official National Formulary, or any supplement to any of them; and (B) articles intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease in man or other animals; and (C) articles (other than food) intended to affect the structure or any function of the body of man or other animals; and (D) articles intended for use as a component of any article specified in clause (A), (B), or (C)''.[2] A dietary supplement is a special category of food and is defined as: ''a product (other than tobacco) intended to supplement the diet that bears or contains one or more of the following dietary ingredients: a vitamin, mineral, amino acid, herb or other botanical; or dietary substance for use to supplement the diet by increasing the total dietary intake; or concentrate, metabolite, constituent, extract, or combination of any ingredient described previously''.[2]

Canada's Food and Drugs Act defines drugs as ''any substance or mixture of substances manufactured, sold or represented for the use in a. the diagnosis, treatment, mitigation or prevention of a disease, disorder or abnormal physical state or its symptoms, in human beings or animals, or b. restoring, correcting or modifying organic functions in human beings or animals, or c. disinfection in premises in which food is manufactured, prepared or kept''.[3] Under Canadian legislation there exists a sub-class of drugs defined as Natural Health Products (NHP).[4] NHPs are drugs that are considered to be ''herbal remedies, traditional and homeopathic medicines and materials derived from plants, algae, bacteria, fungi or non-human animal material, amino acids, essential fatty acids, probiotics, minerals, several vitamins and synthetic duplicates of the natural ingredients''.[4] Despite widespread use of the terms in the research community, in marketing, and in other countries, there are no regulatory categories called functional food or nutraceutical in the US or Canada. For the purposes of this chapter, nutraceuticals will refer to natural health and food products that fall under conventional foods, Canadian NHPs and US dietary supplements.

5.1.2 Definition of Bioactives in the Context of Natural Health and Food Products

The Merriam-Webster Dictionary defines the word bioactive as an adjective meaning ''having an effect on living organisms''.[5] Given the breadth of this definition, this chapter will focus only on the narrower definition of constituents of functional foods and nutraceuticals. A discussion of bioactive phytochemicals that are potent pharmacophores being used as drugs to treat serious conditions (atropine, cocaine, digoxin, morphine, paclitaxel vincristine, to name a few) is beyond the scope of this chapter and the definition will further be limited to bioactivity in humans. Bioactivity can work in more than one direction; there are substances that have harmful effects, those that have salubrious effects and in many cases a substance can have both effects. We should be mindful of the words of Phillippus Aureolus Theophrastus Bombastus von Hohenheim (Paracelsus): ''All things are poisons and nothing is without poison; the dose alone makes, that a thing is not a poison''.[6]

Bioactive substances in natural health and food products include macro- and micro-nutrients, such as carbohydrates, proteins, fats, fatty acids, sugars and several vitamins and minerals, that are considered essential to body processes.[7] Over the last several decades, researchers have hypothesized that certain nutrients may confer health benefits over and above prevention of deficiency disease. Examples include eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from fish oil, selenium, beta-carotene, vitamins A, D, E and others. These naturally occurring secondary metabolites are defined as organic compounds that do not appear to be directly involved in the normal growth, development or reproduction of the organism. Modern understanding is that these compounds, include a variety of chemical

classes including carbohydrase inhibitors, lectins, peptides, alkaloids, glucosinolates, and a broad array of phenolic compounds and their glycosides, are not of secondary importance to plants.

On the other side of the spectrum, there are compounds with undesirable bioactive activities that are also of interest in nutraceutical products. These constituents can be inherent to the products themselves and are compounds that can occur in the food supply and botanical NHPs. Alternatively, deleterious compounds can be introduced from external sources arising from contamination such as toxins from weeds, mycotoxins, polyaromatic hydrocarbons, antibiotics, pesticides, and hormones or even artefacts of the processing and manufacturing process.

5.1.3 Potential Health Impact of Food/Nutraceutical Bioactive Constituents

All ingested substances may be bioactive. Even substances that might be considered chemically inert like insoluble fiber in the form of plant cell wall or lignin from ingested plant-derived food can act as a physical stimulant thus promoting gut motility or modification of the microbial ecology of the gut. Plants biosynthesize a large variety of secondary metabolites that have the potential to exert bioactivity when consumed. Alkaloids like caffeine, theophylline, and theobromine are present in coffee, tea, and chocolate. Protoalkaloids like octopamine, tyramine, *N*-methyl tyramine, and *p*-synephrine occur naturally in bananas and citrus fruit, while the cytochrome P450 CYP 3A4 inhibitor 6′7′-dihydroxybergamottin occurs in grapefruit juice. Cassava (manioc, *Manihot esculenta*) contains sufficient amounts of the cyanogenic glycoside linamarin to be toxic unless properly processed prior to consumption. Even soybean (*Glycine max*) contains the anti-nutrients phytic acid and Bowman–Birk protease inhibitor along with the flatulence causing stachyose and the more familiar isoflavone glucosides daidzin and genistin. Terpenoids, phenols, and alkaloids constitute the three most important groups of secondary compounds.

In addition to nutrients that might provide beneficial activity beyond nutrition and the small subset of examples above that exert previously observed biological activity in humans, plants contain a large number of secondary metabolites currently being investigated for potential health-promoting activity. Research on the health effects of these plant bioactives has often stemmed from either traditional medicinal use or epidemiological evidence that dietary intake of foods rich in specific phytochemicals may have positive effects on human health.[8–10] There is a history of studies on plant bioactives leading to the successful development of pharmaceuticals from plant sources,[11] however in contrast to a drug-development pathway, studies based on traditional uses or epidemiological data of food consumption rarely begin with a precise understanding of the bioactive molecules or their mechanisms of action. As such, the state of the science for most products and disease endpoints remains in its infancy, as investigators

try to turn epidemiological observations on the role of diets rich in phytochemicals into concrete information about their role in disease prevention.[12–15] Further, a growing number of recent studies on natural products have reported finding a complex mixture of bioactives that act on multiple targets.[16–18]

5.2 Analytical Testing of Nutraceuticals and Functional Foods

The American Chemical Society defines analytical chemistry as the science of obtaining, processing and communicating information about the composition and structure of matter.[19] In the context of this chapter, the primary focus is measuring the amount(s) of bioactive substances of interest as they occur in the matrix where they are naturally found. There are a variety of reasons for testing, ranging from authentication of raw materials/ingredients to assessing the consistency, strength, and safety of a product. Both desirable and undesirable constituents should be considered, as the former can be used as indicators of quality and possible efficacy whereas the latter can provide information on the safety of the product and a means of detecting adulteration.

The fundamentals of analytical testing for nutraceuticals and functional foods are not that different from those used in conventional food and drug products as the product formats and analytes of interest share many similarities. Methods however, are not universally interchangeable and it is imperative that any analytical method selected is valid and fit for its intended purpose. Testing to product specifications allows manufacturers to evaluate products against their own quality and safety standards, demonstrate compliance with current good manufacturing practices (cGMP), and allows regulators to enforce labeling requirements. Further knowledge of the nutraceutical product composition, including verification of identity and purity, is an important pre-requisite for rigorous scientific study and essential for researchers and policymakers in making public health recommendations.

5.2.1 Identification Methodologies

In order to identify botanicals unambiguously, the manufacturer should follow Good Agriculture and Collection Practices (GACP) for plant identification.[20] Plant identity and authenticity are determined through diagnostic morphological and anatomical features. Such approaches remain useful when raw materials are traded as minimally processed biomass.[21] Unfortunately, ingredients passing through modern ingredient supply chains seldom meet such criteria as manufacturers may deal in highly pulverized plant powders or extracts and manufacturers have come to rely on chemical identification methods instead.[22,23] It is important to understand that chemical methods for identification, including DNA, are only surrogates for true plant authentication and the fitness of such methods will very much be

dependent on the botanical reference materials employed to develop and validate the methods.[21,23,24]

5.2.1.1 DNA Authentication

DNA testing has been purported to be a universal technique for taxonomic identification despite its limitations when applied to plant and herbal supplement identification. The various DNA techniques and approaches are still evolving and the selection of inappropriate methods for particular samples and issues with data interpretation have led to controversy.[25–27] Botanical authentication methods targeting DNA loci must ensure that the genetic regions targeted are unique and specific to the plant species of interest. The challenge is that taxonomy of many plant species is dynamic, under constant revision and not genetic-based; often the genetic regions selected for species differentiation may vary from closely related species by only a single base-pair substitution.[28,29] Additionally, for many finished products the use of DNA barcoding is not possible as the processing methods used may have degraded the DNA to the point where it is no longer a viable tool for identification.[30,31] Some examples where DNA methods have been successfully utilized to authenticate products include lavender honey,[32] *Angelica sinensis*,[33] Garcinia,[34] Echinacea,[35] ginkgo[36] and black cohosh.[31]

5.2.1.2 Targeted and Untargeted Chemical Approaches

There are two main approaches for chemical authentication of botanicals: targeted and untargeted. Targeted methods of analysis, which focus on a specific or limited set of known chemical constituents, is based on the assumption that the specific constituent(s) are unique or at least diagnostic of the plant species. An example would be determination of ginsenoside retention factor (Rf) as being diagnostic for confirming *Panax ginseng* over *Panax quinquefolius*.[37] An inherent limitation of this approach is the lack of diagnostic species-specific phytochemical constituents and the common use of ubiquitous phytochemical markers leading to an inability to detect adulteration and assign identity with confidence.[23,38,39] For this reason a combination of targeted analytical methods would likely be necessary to ensure the identity of a botanical material, in particular with the changing landscape of potential adulteration.[39,40] A further discussion of targeted methods of analysis is found in Section 4.2.3 for marker and/or active constituents.

An untargeted approach whereby the phytochemistry of the material is fingerprinted in an attempt to capture the overall chemical composition or metabolome is often referred to as metabolomics.[41,42] Typically, plant metabolomics data generates >500 000 observations and this wealth of data can be both a blessing and curse, as the validity of the data interpretation will depend on how the data are managed.[43] The key factors in using plant metabolomics data effectively are the experimental design and the statistical analyses conducted.

Chemometrics is the sub-discipline of analytical chemistry devoted to maximizing the information gained from chemical experiments and combines multivariate statistics with mathematical modeling of the data. The use of chemometrics facilitates the identification of chemical patterns or features within the multitude of metabolomics data obtained and enables classification and/or comparison across botanical materials. Validation protocols for chemometric analyses are currently being developed by researchers in this field; however, there is currently no clear consensus on these guidelines. López *et al.*[44] and Cuadros-Rodriguez *et al.*[45] have summarized several tutorials on validation of qualitative methods with chemometric analysis based on sensitivity and specificity and, more recently, the United States Pharmacopeia (USP) published a proposed guidance document on developing and validating non-targeted methods for adulteration detection.[44–46]

The AOAC International has published recommended protocols for the validation of chemical identity methods.[47,48] The utility of these protocols has been illustrated for differentiation of ginseng species by applying ANOVA to spectral fingerprints (UV, near-infrared (NIR), and mass spectrometry (MS)) acquired from three Panax species and soft independent modeling of class analogy (SIMCA) modeling of flow injection MS (FIMS) data from two Panax species, respectively.[49,50] This approach was also demonstrated for differentiation of *Hydratis canandensis* from common herbal adulterants using FT-NIR coupled with partial least-squares (PLS) regression, SIMCA and moving window principal component analysis (MW-PCA).[51] To ensure the methods were valid, large numbers of authenticated test samples were used in these studies including both biological and technical replicates. The acquisition of large numbers of authentic specimens can be a daunting task, so validation of botanical identity protocols can be a lengthy and expensive proposition.[21]

A myriad of chemical techniques, such as those mentioned above, can be employed to generate a phytochemical fingerprint or metabolome for botanical materials or a nutraceutical product. The most commonly employed analytical platforms for botanical-based health products are high performance thin-layer chromatograph (HPTLC), MS paired with separation methods such a high-performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR) spectroscopy and NIR. A brief description of these techniques is provided in the following sections.

5.2.1.2.1 HPTLC Fingerprinting. HPTLC provides superior separation efficiency over conventional thin-layer chromatography allowing for the determination of precise retention factor (Rf) values and effective direct visual comparison of chromatograms. Although, direct visual inspection of bands is typically used for species differentiation, the HPTLC technique can be coupled to densitometric detectors that can generate both quantitative and qualitative data that can be used in chemometric modeling. HPTLC methods can be used for quality control and species differentiation in a variety of herbal products and ingredients. Recent published

examples include authentication of *Rhodiola* spp, discrimination of *Apiaceae* species and differentiation of St. John's Wort from counterfeit materials.[52–54] The information published by Reich *et al.* in 2008 on the validation of HPTLC methods for identification of botanicals is a useful resource.[55] Overall, the proper interpretation of HPTLC data requires significant experience and skill; however, as detection and recording techniques evolve, more objective instrumentation and statistical means of interpretation has developed.

5.2.1.2.2 Chromatography with UV or MS Fingerprinting. Liquid chromatographic separation coupled with UV or MS detection is common for fingerprinting botanicals and when coupled with chemometric analyses can be applied to authentication and detection of adulterants.[56] The chemometric models generated from metabolomic data sets include principle component analysis (PCA), partial least squares discriminant analysis (PLSDA), soft independent modeling of class analogy (SIMCA) and linear discriminant analysis (LDA).[23] These statistical models allow for the evaluation of large data sets or specific portions of large data sets derived from the spectral data to differentiate samples into classes or groups based on similarities and differences observed from the data. When utilizing these methods, it is imperative that the proper pre-treatment techniques, including alignment, scaling, binning, and normalization, are applied to the data sets to account for data variations.[57,58] The success of these models depends largely on the number and diversity of authenticated reference materials, sampling techniques and data acquisition parameters. Some examples of the application of chemometric models to HPLC–UV data include SIMCA to distinguish *Gingko biloba* raw materials and supplements from adulterated products, PCA to differentiate medical cannabis and investigate the impact of domestication on chemical diversity and PCA, PLSDA and support vector machine classification models for authentication of milk.[59–61]

5.2.1.2.3 NIR Fingerprinting. Upon publication of the US Dietary Supplement GMP in 2007, it was the requirement that 100% of the ingredients in a product must be verified for identity by an appropriate, ''scientifically valid'' test method by the manufacturer, which caused considerable consternation. While NIR technology is not new, the application to verifying botanical identity was touted by purveyors of this technique as a robust, time and money saving technique. The initial enthusiasm has waned due to a lack of systematic examples or case studies to show both its limitations and utility. In general, IR methods are rapid and non-invasive techniques that require minimal sample preparation and are high-throughput. However, a significant amount of development is required to set-up the methods, chemometric modeling and data analysis.[23,51] The approach has been most successful with raw materials rather than with finished products or extracts, as it is prone to interferences from a variety of variables.[59,62,63]

5.2.1.2.4 Nuclear Magnetic Resonance. Nuclear Magnetic Resonance (NMR) analytical approaches have several advantages including simple sample preparation, universal detection and reproducibility between laboratories. NMR is traditionally employed for structure elucidation of pure compounds, purity determination and stereochemistry assignment and has recently become a favored data-acquisition technique for metabolomic studies. NMR is much less sensitive than other spectrometry methods and the instrument itself and maintenance can cost considerably more, but it does provide a potential means of quantifying phytochemicals in botanical products without isolation or separation steps. NMR data can also be analysed using discriminate data analysis and it has been shown to be a potentially useful tool for differentiating botanical species as demonstrated by studies on *Vaccinium* and *Crataegus* species.[64,65]

5.2.2 Purity Determination

A detailed discussion of purity tests is beyond the scope of this chapter; however, as noted previously, if a study material had microbial or chemical contamination or was otherwise adulterated (*i.e.*, with APIs or unidentified botanicals) the impact on the planned scientific study would be disastrous. It is recommended that any study material be subjected to independent testing for any reasonably anticipated contaminants or adulterants. Potential contaminants include: (1) substances that pose no health risk except reduction in strength or economic value because an inert material displaces some mass of the active substance (benign non-target plant material, soil, insects, excess water, *etc.*); (2) substances that pose a direct health risk such as toxic non-target plant material, mycotoxins, pesticides, toxic elements, sulfites, allergens, pathogenic microorganisms, residual solvents, non-declared drugs.

In all GMP regulatory environments, manufacturers are required to be familiar with all steps of the manufacturing process at which a hazard (*e.g.* contaminant) might be introduced and control the step. This requires knowledge of the most likely contaminants, the setting of tolerances for those substances, and test methods against which compliance with those tolerances will be evaluated. Under the Canadian Natural Health Products Regulations (Section 44 (2) (a)), finished-product specifications must contain detailed information regarding the purity of the NHP including tests and methods and tolerance limits for microbial contamination, elemental impurities and if relevant to the product mycotoxins, solvent residues, and pesticides.[66] The WHO has published guidelines for assessing quality of herbal medicines with reference to contaminants and residues[67] and international Pharmacopoeias (USP,[68] HKCMMS,[69] EP[70]) address these issues in detail in general chapters and within individual monographs. As with any analytical procedure, test methods must be scientifically valid and suitable for their intended use.

5.2.3 Analysis of Active and Marker Compounds

Another determinant of quality for natural health and dietary supplement products is a specification for potency (strength) or content as determined by measurement of marker and/or active compound(s) or *via* non specific assays of a class of constituents. The assays employed for acceptance of raw ingredients in process control of manufacturing or finished-product release, can vary from a simple extraction with spectrophotometric analysis to complex sample processing followed by chromatographic separation and spectroscopy/spectrometry-based detection methods. Although direct analysis of the specific analytes responsible for salubrious or toxic effects is the ideal situation, such a measurement may not be feasible. In the case of many plant products the identity of the putative active compounds is not necessarily known. Biological effects of a product can sometimes be associated with marker compounds through correlation of their levels with observations of positive clinical or other biological endpoints.

Unless studies designed to establish causality have been specifically performed, only limited associations can be made and the identity of the specific active agent may be unknown. In other cases, the identity of the active agents may well be known but they are unstable or not accessible to existing analytical techniques. As such, it may be more desirable to utilize marker compounds as a means to assess the product or ingredient. The selection, development and validation of methods for marker/active determination is further discussed in Section 4.3.

The conventional methodological approach used to evaluate most macronutrients in food products focus on classes or categories of compounds. For instance, total fat is typically determined using gravimetric methods that quantify all compounds from the sample extracted using organic solvents,[71,72] and total protein is typically determined through determination of total nitrogen in the sample through Kjeldahl or Dumas based methods.[73] Other indirect universal methods are used to quantify specific classes of compounds such as spectrometric methods used to determine total anthocyanins[74] and phenolic compounds.[75]

Historically, interest in phenolic content was based on the associated antioxidant activity. The assumption made was that ingestion of compounds that inhibit oxidation would quench reactive oxygen species (ROS) that would otherwise damage cells. Antioxidant compounds have thus been the subject of a considerable amount of interest to the biomedical research community. At the forefront is the very large category called flavonoids, which were originally singled out due to their purported antioxidant activity but were later found to exhibit other biological activities. For instance, hydroxylated flavonoids like quercetin and naringenin have generally been found to inhibit P450 isozymes *in vitro*, while nonhydroxylated flavonoids (flavone, nobiletin, tangeretin) generally stimulate P450 systems *in vivo* and *in vitro*.[76,77]

The generally favorable public perception of antioxidant activity has ensured that assays purported to measure antioxidant activity continue to be

used in the evaluation of ingredients and products. Numerous methods, often named using an acronym for the reagent used in the assay, exist including: 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid (ABTS), cupric reducing antioxidant capacity (CUPRAC), 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH), Folin–Ciocalteu (FC), ferric reducing ability of plasma (FRAP), oxygen radical absorbance capacity (ORAC), total antioxidant capacity (TAC), Trolox equivalent antioxidant capacity (TEAC), and total peroxyl radical-trapping antioxidant parameter (TRAP).[78] In general, these are all *in vitro* methods that measure the oxidative degradation of a compound in the presence of the sample of interest through colorimetric measurements at set intervals of exposure. Unfortunately, the similarities of these methods does not equate to equivalence of results. The International Union of Pure and Applied Chemistry (IUPAC) and AOAC International both reported that the results cannot be compared because the methods are based on different mechanisms, redox potentials, and are susceptible to pH, solvent, and sample matrix effects.[79,80] IUPAC concluded ''that no single 'universally' accepted assay is adequate in itself to precisely and quantitatively detect/determine all actions of a putative antioxidant''.[79]

Furthermore, 'antioxidant' activity has seldom been observed *in vivo* and at least two journals have recently implemented policies to reject without review papers that employ antioxidant and total phenolic assays and that focus primarily on these assays. In 2012, the US Department of Agriculture (USDA) removed its ORAC database ''due to mounting evidence that the values indicating antioxidant capacity have no relevance to the effects of specific bioactive compounds, including polyphenols on human health''.[81] In 2011, AOAC International concluded that ''antioxidant'' is primarily a marketing tool.[80] Perhaps the greatest limitation of antioxidant and indirect assays in general, is the reliance on surrogate markers of bioactivity that are not necessarily correlated to the desired biomedical effect. This over-reliance on surrogates or markers can be misleading with respect to bioactivity when conducting studies to the extent that feasible products should be comprehensively characterized.

Non-specific methods are also prone to being fooled through interfering substances and/or adulterants as demonstrated by the highly publicized reports of protein products adulterated with melamine.[82] Along with the increasing demand for turmeric powder, instances of economic adulteration with metanil yellow (sodium **3-[4-anilinophenylazo] benzenesulfonate**), which is a synthetic, non-permitted food color and additive;[83,84] lead chromate,[85] and acid orange 7 (sodium 4-[(2*E*)-2-(2-oxonaphthalen-1-ylidene) hydrazinyl]benzenesulfonate) (Dixit *et al.*[83]) have been reported. It is recommended that non-specific methods, such as spectrophotometric, be avoided in favor of a specific analysis, such as measuring curcuminoids directly, to avoid adulterated products.[86]

5.3 Method Development and Validation

Many of the methods used for analysis of natural health products and dietary supplements are derived from methodologies originally designed for

analysis of whole plant parts. These methods are often low-throughput and not suitable for routine ingredient analysis or are unsuitable for finished products that bear little resemblance to the original plant biomass.[87] As noted above, ingredients seldom resemble the original plant material, as the modern ingredient supply chains more commonly provide highly pulverized plant powders or extracts. It is imperative that any methods selected for use are carefully reviewed to ensure they are not being applied out of their scope and meet all required performance criteria. Methods often require modifications to ensure optimized detection of the target analytes in the complex finished-product matrix. Validation of all analytical methods is the process by which methods are shown to be fit for their intended purpose and is the basis for unbiased data reporting.

5.3.1 Method Optimization

Optimization of an analytical method can be accomplished through several different means and a range of parameters must be considered. Variables such as choice of extraction solvent, length of extraction time, number of extraction cycles, temperature and equipment or technology used, can all effect the efficiency of an extraction. These same considerations must also be addressed for factors that may influence the chromatographic and detection parameters. Given that these products contain a myriad of potentially interfering compounds, optimization studies must be performed on the actual matrix of interest, as opposed to a chemical reference, in order to observe realistic performance. The use of additional clean-up steps or improvements of separation in chromatographic methods may be necessary to ensure that no interference occurs from these agents.

Proper planning prior to optimization can allow for a more efficient workflow and provide a higher probability of achieving precise, accurate methods. The use of factorial designs can greatly assist in identifying those variables that would be most impactful towards method performance, thereby directing the focus towards the evaluation of these variables and ensuring efficient use of time and resources.[88,89] For example, by using a seven factor Plackett–Burman experiment during the development of a method to measure curcuminoids in turmeric, it was identified that the significant factor effecting the extraction efficiency was the concentration of methanol.[86] Additional experiments were then focused on determining the optimal methanol concentration rather than other factors found to be not significant.[86]

The Youden ruggedness trial, also a factorial design, can be performed as part of method evaluation to determine which experimental variables are most sensitive to fluctuations.[90] For example, a Youden trial performed as part of the validation for determination of flavonol aglycones in Gingko materials found that volume of sample hydrolyzed and test sample weight were significant factors affecting results, indicating that these variables must be measured accurately and precisely in order to ensure valid results are obtained with this method.[91]

5.3.2 Method Validation

Analytical method validation is the process through which a method's performance is assessed and fitness for purpose determined. Validation studies ensure that the method being evaluated is suitable for its intended use with respect to detecting, identifying and/or quantifying the components of interest. The level of validation required depends on the intended use, for example methods intended to be used across laboratories would require a multi-laboratory validation study, while a single laboratory validation is sufficient when methods and data are not translated outside of the lab.

Validation guidelines for chemical methods have been published by several organizations including the AOAC,[86] the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH),[92] United States Food and Drug Administration (FDA),[93] and The International Union of Pure and Applied Chemistry (IUPAC).[94] In general, the validation guidelines require demonstration of method applicability, selectivity, linearity, repeatability (precision), accuracy, limits of quantification and limits of detection and stability. Table 5.1 provides a summary of the performance characteristics regarded as most significant for different types of analytical procedures.[95]

Highly precise and accurate methodologies are desirable; however these traits can be dictated by the concentration of analyte being measured. While reviewing collaborative study data, Horwitz *et al.* found that, in general, as concentration of analyte decrease across two orders of magnitude, the relative standard deviation of reproducibility ($rRSD_r$) increases by a factor of two.[96] This relationship was observed regardless of the nature of the analyte or the type of methodology used.[96] Horzitz *et al.* developed a formula through which a predicted relative standard deviation of reproducibility ($pRSD_r$) could be determined based on the concentration of analyte.[96] AOAC

Table 5.1 Validation characteristics regarded as the most important for the analytical endpoint. "−" signifies that this characteristic is not normally evaluated, "+" signifies that this characteristic is normally evaluated, and "±" indicates that this characteristic may need to be evaluated in some cases.

	Type of analytical procedure			
		Testing for impurities		
Characteristic	Identity	Quantitation	Limit	Strength (assay)
Accuracy	−	+	−	+
Precision				
Repeatability	−	+	−	+
Intermediate precision	−	+	−	+
Specificity	+	+	+	+
Limit of detection	−	±	+	
Limit of quantification	−	+	−	−
Linearity	−	+	−	+
Range	−	+	−	+

guidelines utilize HorRat values derived from the ratio of $rRSD_r$ to $pRSD_r$ in the evaluation of method performance with an acceptable range of 0.5–2.0 for single laboratory validation studies.[90]

5.3.3 Method Fitness of Purpose

Whether a method is fit for its intended purpose is dependent on how well it performs under the specified conditions of analysis. Methods that have been subjected to validation studies are only suitable for the specified analytes and matrices included in the validation study. Applying these methods to different products would be out of scope and introducing a new matrix would require a matrix-extension study. For example, in response to consumer product trends, a method validated for Echinacea spp. raw materials,[97] would require an additional matrix-extension study prior to use for testing tinctures and dry extracts in finished products.[98]

Key considerations when selecting, developing, or utilizing a method include an understanding of the following. (1) Study material: what are the origins, how was it processed, what is the format for the final delivery system or dosage form? (2) The compounds being measured: what is the chemical nature of the target analytes and what will be the most appropriate detection technology? (3) Analyte concentration: do you have to be concerned about sensitivity and does the method cover the expected concentration range of the anlayte in the study material? (4) Presence of common excipients and/or any additional ingredients that may impact the analysis: will other analytes present have an impact of the selectivity of the method? (5) Contaminants, and in the case of methods to detect adulteration, the identity and behavior of potential adulterants in the sample.

Ignoring these considerations can have serious consequences for the analysis, interpretation of resulting data and for the final products themselves. In early 2007, severe adverse event reports associated with pet foods, wheat gluten, baby formula and other dairy products imported from China led to the discovery that these products had been adulterated with melanine and cyanuric acid.[82] The addition of non-protein nitrogen compounds to these products appeared to be predominantly motivated by a desire to increase the products' apparent protein level and mask other adulteration activities.[82] These products could no longer be assessed through the commonly used and relatively simple Kjedhal and Dumas based methodologies, but rather required methods that could detect these adulterants.

The FDA published two such methods in 2008: a GC–MS screening method for dry protein materials[99] and a LC–MS/MS method for infant formula.[100] Although these methods are effective at detecting adulterated products, they do not directly quantify protein, thus, additional analysis is required. Several more direct methods for protein quantification exist, including near-infrared methods that measure peptide bonds[101] and methods that quantify amino acids.[102] These direct methods were not typically used as they were considered more expensive and difficult to perform, however

given the concerns about adulteration, these direct methods may now be more appropriate for a quality control setting.

In another example, the commonly performed determination of chondroitin sulphate in raw materials by titration with cetylpyridinium chloride has been found to be unsuitable for products that also contain compounds such as glucosamine, methylsulfonylmethane (MSM) or certain emulsifiers which can interfere with the titration.[103] To determine if interfering compounds are present a purity determination using electrophoresis is required prior to using the titration method.[103] Quantification of chondroitin in samples that do possess interfering compounds would require a more intensive method that uses enzymatic hydrolysis of the glycosaminoglycan polymer followed by LC–UV separation and detection of the parent disaccharides.[104]

The analysis of cranberry ingredients and products is another example of where methods have been employed that are not suited for their intended purpose. Cranberry materials have been found to be adulterated with other lower-cost proanthocyanidin and anthocyanin rich extracts and pigments, such as peanut skins (*Arachis hypogaea*) or other lower-cost anthocyanin rich ingredients including mulberry (*Morus* spp., Moraceae) fruit, hibiscus (*Hibiscus sabdariffa*, Malvaceae) calyx, black bean (*Phaseolus vulgaris*, Fabaceae) skin, or black rice (*Oryza sativa*, Poaceae).[105] Common methods for proanthocynidins measure quantities only and do not differentiate the source of the material, providing no guarantees that the material is indeed cranberry. Non-specific spectrometric methods for anthocyanins also provide no means for source determination and are also susceptible to being fooled by added pigments. Different LC methods that directly measure chemical constituents, such as intact anthocyanins and specific polyphenols, can provide profiles that can be indicative of the plant source and are the preferred methods for identity and purity assessments.[106,107]

5.4 Sources of Methods, Resources, and Best Practices

There is an obvious need for reliable validated analytical methods for nutraceutical products. Methods to analyze these types of products are typically derived from methods used for the evaluation of raw botanicals and can be used as a starting point for method development and optimization. Following this, validation studies on the optimized method need to be done ensuring the method is fit for its intended purpose. There have been several methods optimized and validated for a wide variety of dietary supplement and natural health product components and several methods are listed on the NIH Office of Dietary Supplements' (ODS) Analytical Methods and Reference Materials (AMRM) website located at https://ods.od.nih.gov/Research/AMRMProgramWebsite.aspx.[108] AOAC International has published several AOAC Official Methods of Analysis (OMA) for dietary supplements, the most recent of which are found below in Table 5.2.[109]

Table 5.2 The recently approved AOAC International ''First Action Official Methods of Analysis''. HPLC = high performance liquid chromatography, UV = ultraviolet detection, UV/DAD = ultraviolet diode array detection, UV-VIS = ultraviolet-visible detection.

Material	Constituents	Method	Matrixes	Reference
Aloe Vera	Aloe polysaccharides	Spectrophotometric	Aloe powders and liquid	Metcalfe[116]
Echinacea	Phenolic constituents	HPLC–UV/DAD	Raw materials, dietary ingredients and dietary supplement products	Brown *et al.*[97]
Ginseng	Total ginsenosides	HPLC–UV/DAD	*Panax* spp. root materials, extracts and finished products	Brown *et al.*[117]
Ginger	Gingerols and shoagaols	HPLC–UV/DAD	Ginger rhizome powder, dry extract, tablets and capsules	You *et al.*[118]
Kava	Kavalactones and flavokavains	UPLC–UV	*Piper methysticum* raw materials, dietary ingredients and dietary supplement products	Liu *et al.*[119]
Chondroitin	Chondroitin sulfate	HPLC–UV	Raw materials and finished products containing chondroitin sulfate	Ji *et al.*[104]
Green Tea	Theanine	HPLC–UV–Vis	*Camellia sinensis* dietary ingredients and supplements	Ofitserova and Nerkar[120]
Turmeric	Curcumin, demethoxycurcumin and bis-demethoxycurcumin	HPLC–UV/DAD	*Curcuma longa* raw material and finished products	Mudge *et al.*[86]
Dietary ingredients and supplements	Phosphodiesterase type 5 inhibitors	LC/Quadrupole-Orbital Ion-Trap MS	Dietary ingredients and supplements	Vaclavik *et al.*[121]
Ashwagandha	Withanolides	HPLC–UV	*Withania somnifera* raw material and dried extract	Koshy *et al.*[122]
Kratom	Mitragynine and 7-hydroxy mitragynine	HPLC–UV	*Mitragyna speciose* raw materials and finished products	Mudge and Brown[123]

Additionally, various national pharmacopeias and international organizations have published methods and quality standards for a variety of botanical products. These include the USP,[68] the European Pharmacopeia,[70] the Hong Kong Chinese Material Medica Standards Office,[69] The Pharmacopoeia of the People's Republic of China[110] and the American Herbal Pharmacopoeia.[111] When using these methods, it is important that the scope and purpose of the method is well understood.

Prior to conducting research studies on nutraceutical products, researchers are strongly encouraged to review the NIH – National Center for Complementary and Integrative Health (NCCIH) Policy: ''Natural Product Integrity'' that is designed to provide guidance to researchers on the information they should acquire for products they intend to study, as compliance will provide the investigator with a measure of confidence that their research will yield definitive and reproducible results.[112] A number of recent reviews have been published that provide specific guidance to researchers intending to study botanicals-based products. Published by Kellog *et al.* in 2019, ''Selection and characterization of botanical natural products for research studies: a NaPDI center recommended approach'' identifies the critical questions researchers must address prior to undertaking preclinical and clinical studies.[113] An excellent overview of the challenges associated with assessing the safety of botanicals-based products, including a discussion of product characterization, was published by Shipkowski *et al.*[114] In September 2018, NCCIH held a transdisciplinary workshop with the goal of more effective bridging from basic natural product research to clinical trials. An outcome of this workshop, currently in preprint[115] is guidance on ways to improve natural product research translation from product source to clinical trial and includes best practices for biomedical researchers intending to conduct studies. Ultimately all of these documents reiterate the need to conduct comprehensive characterisations of the products intended for study, and only methods fit for their intended purpose will ensure that results obtained are truly reflective of quality, safety and/or efficacy of the product or ingredient.

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CHAPTER 6

Encapsulation of Nutraceuticals

APRIL X. XU, ELIZABETH A. L. WEST AND
MICHAEL A. ROGERS*

Department of Food Science, University of Guelph, Guelph, Ontario
N1G2W1, Canada
*Email: mroger09@uoguelph.ca

6.1 Classification of Bioactives and Nutraceuticals

Correlations amongst diet and health are well established, where sub-optimal diets are considered a prevalent and preventable risk factor for many non-communicable diseases and the emergence of dietary-related diseases.[1,2] Considering that ultra-processed foods are the major calorie source in Western society and typically have sub-optimal micronutrient composition and concentration makes bioactive supplementation integral to maintaining a healthy population.[3] This notion has led to rapid growth in the market value of functional foods and nutraceuticals – a portmanteau coined by combining 'nutrition and pharmaceutical'.[4] Unfortunately, bioactives have notoriously low bioavailability (*i.e.*, concentration of bioactives that reach their site-of-action and confer biological effects) and without a mechanism to increase their bioaccessibility (*i.e.*, concentration of bioactive available to be absorbed by the intestinal epithelial layer), they pass through the gastrointestinal tract and are excreted.[5,6] Although nutraceutical supplementation provides wide-ranging potential biological benefits, in order to confer those physiological effects they must first be absorbed and then reach the target site, all the while being maintained in their active form.[7]

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6.1.1 Considerations in Bioactive Delivery

Formulation of delivery vehicles provides a protective barrier between the nutraceutical and the harsh environment of the human gastrointestinal tract (GIT). Encapsulation techniques aim to limit the confounding factors adversely influencing bioaccessibility, absorption, and transformation of nutraceuticals. In some cases, bioactive accessibility is reduced immediately upon exposure to the oral cavity, as both mechanical and enzymatic degradation of the nutraceutical matrix occurs. As well, matrix dissolution begins upon mastication with saliva, which may cause appreciable changes in the nutraceutical composition and structure.[8] Further, upon exposure to negatively charged mucin, positively charged bioactives can non-covalently bind to the mucin reducing bioaccessibility.

The harsh, acidic (pH 1–3) gastric environment introduces additional barriers to bioactive accessibility associated with the presence of gastric fluids, minerals, surface active substances, and digestive enzymes which further break down the nutraceutical matrix.[9] During transition through the gastric compartment, bioactives must avoid chemical and metabolic transformations prior to absorption in the small intestine. In addition, they must avoid inactivation and/or being transformed into potentially unknown metabolites while maintaining the correct dosage.[10,11]

Bioactive absorption occurs at the microvilli of the intestinal enterocytes which maximize the surface area available for transport. Microvilli are covered with a viscous glycoprotein mucosal layer that restricts absorption of large molecules and forms strong hydrogen bonds and hydrophobic interactions with certain bioactives.[8] Transport into enterocytes is mediated *via* active (utilizing transport proteins) and/or passive (through diffusion) mechanisms.[8] Proteins embedded at the brush border (containing microvilli and enzymes (*e.g.*, peptidases, lipases, and amylases that facilitate the degradation and absorption of nutrients)), facilitate transport of small molecules.[8] Many active transporter proteins are known to move bioactives such as some vitamins, peptides, and free fatty acids.[8,12,13] Passive transport between enterocytes restricts the diffusion of hydrophilic compounds due to interactions with the phospholipid bilayers. For example, some fruit polyphenols have notoriously low bioavailability, as their hydrophilic nature reduces movement between epithelial cells.[14,15] Bioactive transport through the selectively permeable cellular membrane is a precursor to absorption and systemic circulation.[16,17]

Following absorption, bioactives enter systemic circulation either *via* the hepatic portal vein or the lymphatic system. Further reductions in bioavailability can occur due to first-pass metabolism where excretion can limit accumulation within tissues where the bioactive confers its biological responses.[18] Lipophilic compounds tend to enter the lymphatic system where they bypass first-pass metabolism, which increases their bioavailability.[19,20] On the other hand, bioactives that enter the portal vein undergo first-pass metabolism because they are transported to the liver where they

are partly metabolized.[20] Metabolic activity reduces bioavailability in most cases; however, some metabolites can still confer the original biological function. However, first-pass metabolism typically reduces activity before the bioactive reaches the systemic circulation.[20] Due to these complex series of physiochemical, physiological, and biochemical events that occur along the GIT, delivery systems for nutraceuticals are attractive to enhance absorption and bioavailability. Unfortunately, the chemical diversity of nutraceuticals means no universal delivery system exists; therefore, each bioactive requires a unique strategy to increase its bioavailability and target delivery. In an attempt to differentiate challenges and limitations for bioactive delivery, two classification schemes are commonly used: the Biopharmaceutics Classification Scheme (BSC) and Nutraceutical Bioavailability Classification Scheme (NuBACS).[18,21]

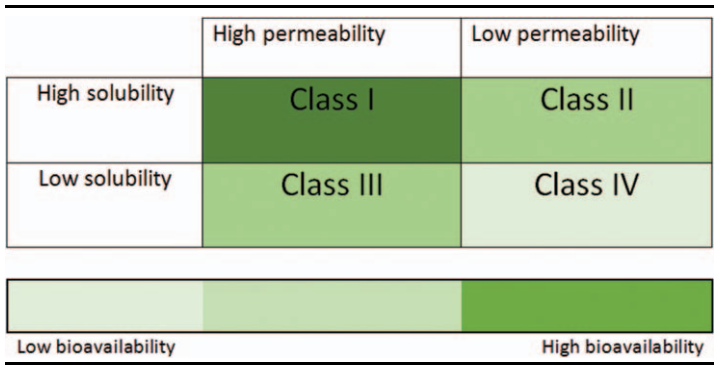
6.1.2 Biopharmaceutics Classification Scheme (BCS)

In pharmaceutics, bioavailability of active compounds is classified using the Biopharmaceutics Classification Scheme (BCS).[22] BCS is a tool that correlates *in vitro* dissolution with *in vivo* bioavailability (IVIVC) for oral delivery.[21,22] A successful correlation using sensitive and reproducible dissolution data obtained from defined physicochemical and hydrodynamical conditions can be used as a surrogate for bioequivalence, reducing costs and unnecessary human testing during drug development.[23,24] Official methods utilize USP (United States Pharmacopeia) Apparatus 1 (basket attachment) and/or USP Apparatus 2 (paddle attachment) in media, such as simulated gastric and intestinal fluids (fasted state or fed state), to perform dissolution studies that are used to approximate *in vitro* activity of dosage forms in standardized solutions.[25–27]

BCS categorizes bioactives based on physiologically relevant factors central to assessing bioavailability of solid oral drugs.[22,28] Indeed, bioactives are categorized into four major classes based on their solubility and intestinal permeability characteristics (Table 6.1). Class I are highly soluble and have high permeability;[21,29] Class II have low solubility but high permeability;[18,21,29] Class III are highly soluble but have low permeability;[21] and Class IV bioactives have the lowest bioaccessibility due to their low permeability and low solubility.[21,29]

The bioavailability of Class I bioactives, which include theophylline and caffeine, are not limited by luminal solubility nor enterocyte uptake; however, they may be limited by factors not considered under the BCS (*e.g.*, liberation, interactions, chemical transformation, first-pass metabolism, and efflux transport).[18,21,30] Although delivery systems are not required to enhance uptake, many applications for delivery systems for Class I bioactives include masking bitter flavors, controlling or prolonging release rates, and/or providing protection during manufacturing, processing/storage, and/or digestion. For instance, caffeine encapsulated in alginate hydrogel beads coated with chitosan showed extended release profiles and bitterness

Table 6.1 Biopharmaceutics Classification Scheme (BSC) to assess bioavailability of nutraceuticals based on dissolution and permeability.



masking (bitterness intensity score drops to 2 for encapsulated caffeine compared with 6.5 for caffeine solutions).[31] Another example is encapsulation of theophylline into interpenetrating polymer network microspheres of chitosan and methylcellulose, which extends release profiles (ranging from ~60–80% release after 12 hours) that can be tailored using different polymer ratios and degree of crosslinking.[32] Therefore, Class I bioactives still benefit from encapsulation.

Class II bioactives are poorly soluble in aqueous environments and while their low solubility limits bioaccessibility they are not limited by intestinal uptake and permeability. Class II bioactives are typically lipophilic bioactives such as: coenzyme Q10, oleanolic acid, *Pueraria lobata* isoflavones, lutein, and α-tocopherols.[18,21,29,30] The *in vivo* rate-limiting step for Class II bioactive bioavailability is dissolution due to their lipophilicity.[21] Therefore, lipophilic compounds are often encapsulated in gelatin or other water-soluble materials to increase solubility and hence bioavailability.[33] As an example, lutein (a carotenoid) has a solubility of 0.00 ± 0.00 $\mu g\,mL^{-1}$, 0.10 ± 0.03 $\mu g\,mL^{-1}$, and 0.11 ± 0.15 $\mu g\,mL^{-1}$ in water, simulated gastric fluids, and simulated intestinal fluids, respectively at 37 °C.[34] Micro-encapsulation using a water-soluble starch encapsulant increased lutein solubility and hence bioaccessibility by 140% in mice.[35]

Class III bioactives are characteristically not hindered by solubility, but have low bioavailability due to limited permeability.[21] Most phytochemicals, including ellagic acid and catechins reside in this category.[30] Catechins are large, highly-hydrophilic molecules and they cannot pass the lipophilic epithelial layer easily in their raw form. Their bioavailability is improved using chemical modification techniques or when co-administered with bioactives that increase membrane permeability.[29,30] Several encapsulation strategies improve Class III bioactive permeability: inclusion of prodrugs, permeability enhancers, and more recently, nanoencapsulation.[36] Nanoencapsulation using natural polymers such as albumin or lipid-based agents such as liposomes are

commonly employed[36] to increase cellular penetration due to passive diffusion of nanoparticles.[36] Hydrophilic Class III bioactives can also be encapsulated using polymeric micelles with hydrophilic and lipophilic domains, which further enhance permeability by increasing passive diffusion.[36]

Class IV bioactives, which have limited solubility and permeability, include curcumin and soy isoflavones (genistein and daidzein) and are the most problematic due to their lower *in vivo* bioavailability.[21,29,30] This class requires efficient delivery systems in order to confer their physicochemical effects.[37] For example, curcumin's poor solubility ($\sim$2.0 mg L^{-1}) increased 40-fold (77 mg L^{-1}) when encapsulated in hydrophilic gelatin microspheres.[38] A 13-fold increase in *ex vivo* intestinal permeability of curcumin was also observed when encapsulated with tocophersolan-stabilized lipid nanocapsules.[39] Encapsulation techniques of Class IV bioactives enhance their bioavailability by overcoming their low solubility and permeability during digestion and absorption in the GIT.

In the pharmaceutical industry, categorizing bioactives with the BCS has been tremendously beneficial in biowaivers, allowing the bypass of human trials, by categorizing major factors influencing oral bioavailability such as solubility and permeability. However, many factors that influence bioavailability of nutraceuticals are unaddressed in the BCS.[18] For example, BCS does not account for metabolic processes such as first-pass metabolism, chemical and metabolic transformation during digestion, release of the bioactive from its matrix, interactions of the bioactive with its matrix or environment, and other mechanisms of absorption.[18] Curcumin has low solubility and permeability but also undergoes extensive first-pass metabolism and biotransformation during digestion, which both adversely affect bioavailability and are unaddressed in the BCS.[40,41] In addition, release of vitamin E from cubed almonds is significantly different ($P < 0.005$) than from finely ground almonds, suggesting that the food matrix plays a key role in nutraceutical bioavailability.[42] These gaps in the BCS call for a modification when classifying nutraceuticals.

6.1.3 Nutraceutical Bioavailability Classification Scheme (NuBACS)

Although BCS has been useful in designing pharmaceutical delivery systems in general, it has limited applicability for nutraceuticals due to complexing factors such as degradation, biotransformation, and others factors influencing absorption.[43–45] Due to limitations of the BSC scheme when applied to nutraceuticals, McClements *et al.*, developed the Nutraceutical Bioavailability Classification Scheme (NuBACS) (Table 6.2), as an extension of the original BCS to more robustly classify oral delivery of nutraceuticals.[18] NuBACS accounts for three limiting factors (bioaccessibility (B), absorption (A), and transformation (T)) caused by either the bioactive directly or related to the food/delivery matrix of the bioactive. Each limiting factor is used to define subclasses.[18]

Table 6.2 Nutraceutical Bioavailability Classification Scheme (NuBACS).[18]

B* Bioaccessibility	L: Liberation S: Solubilization I: Interactions
A* Absorption	ML: Mucus layer TJ: Tight junctions BP: Bilayer permeability AT: Active transport ET: Efflux transport
T* Transformation	C: Chemical degradation M: Metabolism

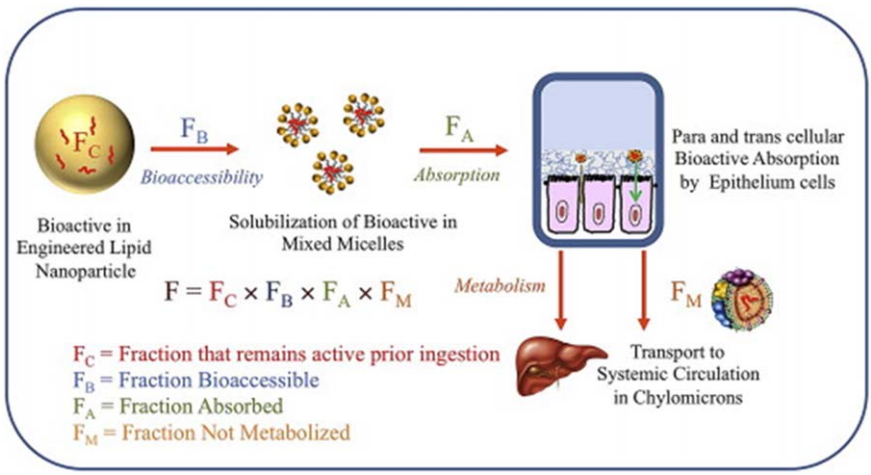


Figure 6.1 Processes in the gastrointestinal tract that alter total bioavailability including solubilization, absorption, and metabolism.
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Bioaccessibility (F_B), the precursor of bioavailability (F), assesses only the fraction of bioactives available to be absorbed (F_A) and must not be metabolized or transformed into an inactive form (F_M) (Figure 6.1). Bioaccessibility accounts for the biophysics of the release phenomenon, which is dependent not only on solubility but also degradation kinetics of the delivery vehicle (*i.e.*, food matrix or encapsulate). In the case of hydrophilic bioactives, bioaccessibility is defined as the fraction solubilized in intestinal fluids, whereas for lipophilic molecules bioaccessibility represents the fraction solubilized within mixed micelles. For a bioactive to be considered highly bioaccessible, at least 75% of the bioactive must be available for absorption and uses the NuBACS designation of B(+). When bioaccessibility is a limiting factor in bioavailability, B(−) is assigned and is then further subdivided into three subclasses: liberation (L), solubilization (S), and

interactions (I).[18] A liberation-limited nutraceutical is inaccessible because it does not get released from its matrix and bioaccessibility is low. These include vitamins and phytochemicals entrapped in cellular structures and inappropriately encapsulated bioactives.[46] Interaction-limited nutraceuticals can also form complexes with other constituents from the food matrix (*i.e.*, positively charged chitosan can form complexes with anionic lipids and disrupt emulsion stability) or with molecules present in the luminal tract that are ubiquitously present during digestion (*e.g.* proteins, enzymes, phospholipids, bile, *etc.*).[47] Thus, depending on the bioaccessibility limiting factor (L, S, or I), the designation of a low-bioaccessibility nutraceutical may be $B(-)_L$, $B(-)_S$, $B(-)_I$ or any combination thereof.[18]

After the bioactive is liberated from the food matrix or delivery vehicle and solubilized in GI fluids, the bioactive must then be absorbed to perform its biological function. Thus, the next limiting factor of NuBACS is absorption. Absorption is defined as the bioactive fraction capable of passing through the mucosa and epithelial layer and available to enter systemic circulation. Nutraceuticals are designated A(+) for highly absorbable nutraceuticals. The absorption-limited class (denoted: A(−)) are subclassified into: mucin layer-limited (ML), tight junction-limited (TJ), bilayer permeability-limited (BP), active transporters-limited (AT), and efflux transporters-limited (ET).[18] A nutraceutical whose absorption is limited by mucin layer transport (ML) is incapable of crossing the mucosa lining of the epithelial layer. These are large compounds (*i.e.*, bioactive peptides, >400 nm) that, in some cases, form strong noncovalent interactions with the sulfated mucin layer.[46] Nutraceuticals with low membrane lipid bilayer permeability (BP) due to polarity, charge, or size may pass through tight junctions (TJ); however, the particles must have a radius <0.7 nm in order to diffuse through the narrow channels between epithelial cells. Bioactive absorption may be limited by passive transport when the bioactive is not able to passively diffuse through the non-polar membrane. Nutraceuticals are only able to pass the membrane bilayer through active transport (AT) if it has an active transport mechanism. Additionally, bioactive absorption may be limited by efflux transporters (ET) that move bioactives into the lumen after crossing the epithelial layer.[18] The designations of absorption-limited nutraceuticals are $A(-)_{ML}$, $A(-)_{TJ}$, $A(-)_{BP}$, $A(-)_{AT}$, $A(-)_{ET}$, or any combination thereof.[18]

The final classification in the NuBACS accounts for chemical transformations (T) rendering bioactive to inactive forms. Bioavailability of this class of nutraceuticals is limited because of changes to their chemical structure resulting in an inactive molecule. Classifications of T(+) are given to highly stable bioactives resistant to alterations in their chemical structures (to be classified T(+) > 75% of the nutraceuticals must remain bioactive).[18] Two subclasses of transformation-limited T(−) nutraceuticals include: chemical degradation-limited (C) and metabolism-limited (M). Nutraceuticals whose bioavailability is limited by transformation undergo chemical degradation (*e.g.*, oxidation, reduction, hydrolysis, or other mechanisms in the food matrix or during digestion) generating inactive metabolites. Metabolism-limited

absorption arises when certain enzymes are deficient, leading to inadequate breakdown of the nutraceutical, and accounts for the decreased concentration of bioactives that occurs as a result of first-pass metabolism.[18] The designations of transformation-limited bioactives are $T(-)_C$, $T(-)_M$, or their combination.

The NuBACS system has been applied to curcumin ($B(-)_{L,S}$, $A(-)_{TJ,AT,ET}$, $T(-)_{M,C}$), epigallocatechin gallate ($B(-)_{L,S}$, $A(-)_{TJ,AT,ET}$, $T(-)_{M,C}$), resveratrol ($B(-)_S$, $A(-)_{ET}$, $T(-)_{M,C}$), and carotenoids ($B(-)_{L,I,S}$, $A(-)_{TJ,AT,ET}$, $T(-)_C$).[18] Carotenoids are given a $B(-)$ designation since, depending on the food matrix, they can be complexed to proteins in plant plastids.[46,48,49] Bioavailability of carotenoids is low because they self-associate and form complexes with soluble fibers and phytosterols; their inherently low solubility further results in low oral bioaccessibility.[18,49–52] Carotenoid bioavailability is further limited by several absorption factors. For example, tight junction transport is limited because they often form aggregates or are contained in mixed micelles and are too large to pass.[49] Active transports for carotenoids are easily saturated, which may also be a limiting factor, and intracellular carotenoids may be transported back into the lumen through efflux transporters.[18,49,53] Finally, carotenoid bioavailability is limited by chemical degradation, as it undergoes rapid oxidation by metal ions, singlet oxygen, and heat[18] (Figure 6.2).

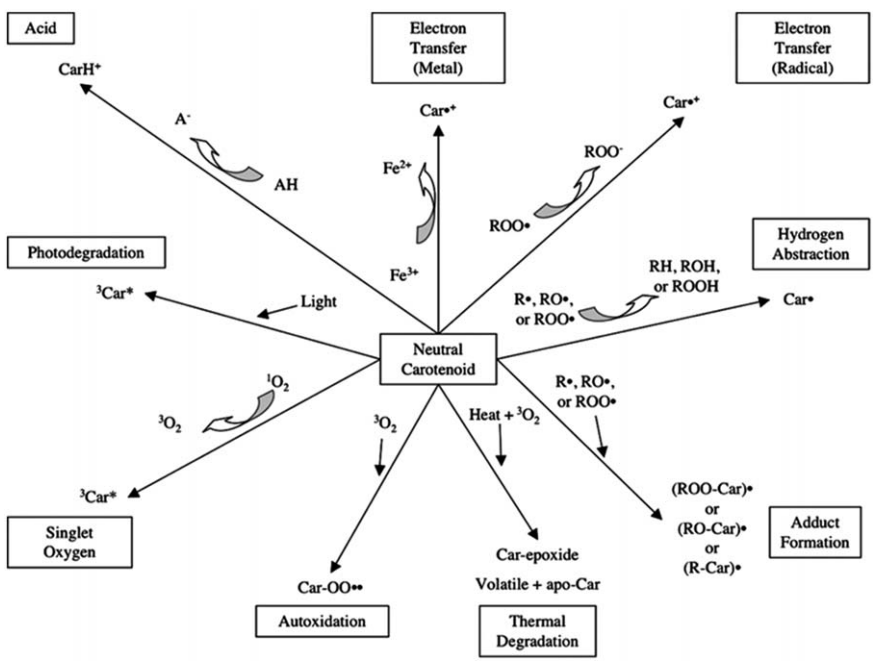

Figure 6.2 Potential transformation reactions for carotenoids.
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6.2 Benefits of Bioactive Encapsulation

The NuBACS classification system outlines obstacles that need to be overcome to improve bioavailability. Encapsulation techniques are commonly used to overcome these obstacles by protecting bioactives from their environmental conditions and limit deteriorative chemical reactions (*i.e.*, overcome transformation (T) limitations). Moreover, encapsulation increases bioaccessibility and absorption (B, A) by increasing GIT solubility (B), increasing intestinal mucosa and membrane permeability (A), and facilitating site-specific delivery (A). Unpleasant flavors and/or odors associated with some nutraceuticals are masked when properly encapsulated. As nutraceuticals differ drastically in terms of chemical structure and function, a wide variety of encapsulation techniques, as well as encapsulant materials, have been investigated in the design of optimal delivery systems for specific bioactives.

6.2.1 Chemical and Environmental Protection

Nutraceuticals are frequently isolated and purified from food and either added back at higher concentrations to compensate for bioactives reduction during processing, added to new functional foods, or formulated into capsules or tablets. Isolated nutraceuticals can lose their chemical stability (*i.e.*, through degradation, isomerization, *etc.*) and thus require protection from storage and/or harsh luminal conditions.[8,54–59] Through encapsulation, bioactives are protected until they reach their release site. Bioactives containing conjugated double bonds (*i.e.*, lycopene, tocotrienol *etc.*), are notoriously unstable and require encapsulation; conjugated double bonds are essential to the bioactive's antioxidant and free radical quenching ability. These bonds are highly reactive, unstable in acidic environments, and susceptible to oxidants, light and heat that reduce bioactivity *via* chemical degradation.[56,57]

Lutein and lycopene contain 8 and 11 conjugated bonds, respectively. In an effort to protect lycopene from degradation it was mixed with Tween 80, ethanol and water and was encapsulated in *Chlorella pyrenoidosa* cells (CPC).[56] Thermogravimetric analyses showed that a 600 °C heat treatment reduced unencapsulated lycopene by 83.02% and CPC-encapsulated lycopene by 80.64%.[56] Encapsulation also protects bioactives against oxidative and light degradation during storage.[56,57] Pu and Tang illustrated that CPC-encapsulated lycopene prevented degradation over a 60-day accelerated shelf-life study at 4, 25, and 35 °C by ~30, 58, and 60%, respectively compared with unencapsulated lycopene.[56] The antioxidant activity of lycopene, as measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, was decreased by ~46% after 25 days at room temperature in natural light; however, CPC-encapsulated lycopene decreased by only ~3% under those conditions.[56] In addition, lutein extracted by supercritical CO_2-extraction from durum wheat bran oil was stable for 22 days but decreased by 20% after 30 days at 4 °C.[54] Lutein encapsulated in sodium alginate beads

did not degrade during the 30-day storage period and decreased by only 27% after 90 days at 4 °C.[54] Similarly, vitamin E and quinones, also present in the durum wheat bran oil, have lower concentrations of hydroperoxides (an indicator of quality degradation in oils) after encapsulation in sodium alginate beads. Unencapsulated oils had ~5- and 12-fold more hydroperoxides than their encapsulated counterparts at 4 °C and room temperature, respectively. Similar trends were observed after 90 days under the same storage conditions.[54] α-Tocopherol concentration in sodium alginate encapsulated oils follows similar trends: bioactive concentration was 90% after 30 days, 50% after 60 days, and 20% after 90 days; unencapsulated α-tocopherols were degraded to a greater extent (72% after 22 days and 32% after 30 days) under identical conditions.[54]

Encapsulation protects nutraceuticals such as polyphenols and antioxidants from environmental and chemical degradation; enhances stability during storage, distribution and processing; decreases metabolism of the bioactive; reduces transformation-limited bioavailability; and facilitates targeted delivery to optimize physiological benefits.[55,58]

6.2.2 Increasing Solubility and Bioaccessibility

Insoluble nutraceuticals are ineffective at reaching their target sites, as they are not absorbed and are primarily excreted. These nutraceuticals, with B(−) in NuBACS, require delivery systems to improve dissolution throughout the GIT.[18,60] Encapsulation potentially increases bioaccessibility of insoluble nutraceuticals by modifying polarity with water-soluble encapsulants or by modifying their physical structure from crystalline to amorphous. Curcumin extracted from turmeric has limited applications because it is a very lipophilic molecule and its low solubility translates to low bioaccessibility.[38,57,61] Encapsulating curcumin in liposomes with and without pluronics (copolymers of hydrophobic and hydrophilic polypropylene oxide components) significantly altered *in vitro* bioaccessibility ($P < 0.05$) from 26.9% in liposomes (without pluronics) to >32.7%.[57] Other researchers encapsulated curcumin using gelatin microcapsules that resulted in a 40-fold increase in solubility (2.0 ± 1.3 mg L^{-1} for unencapsulated compared with 77.2 ± 7.7 mg L^{-1} for encapsulated) and an 11-fold increase in bioaccessibility (0.23 ± 0.02 μg/100 μg to 2.63 ± 0.07 μg/100 μg).[38] The increased solubility and bioaccessibility was attributed to the hydrophilic gelatin shell and alterations in the physical state of curcumin from crystalline to amorphous caused by interactions with gelatin post-encapsulation.[38]

6.2.3 Enhancing Stability and Bioavailability

Numerous bioactives classified as T(−) cannot be formulated into functional foods or nutraceuticals in their free form because of their instability throughout the GIT and susceptibility to chemical and/or metabolic transformation.[10,11,18] Resveratrol, a natural polyphenol in grape skin, has had

few applications because of its low bioavailability (<5%).[7,11] It is rapidly metabolized in intestinal enterocytes and by first-pass metabolism in liver where glucuronidation and sulfate conjugation of its phenolic groups diminishes its bioavailability.[11] Resveratrol encapsulation reduces first-pass metabolism allowing it to reach target sites unaltered.[7,11] Casein has been used as an encapsulant to reduce metabolic degradation of resveratrol prior to absorption.[7,11] In rats, oral administration of resveratrol delivered in casein nanoparticles resulted in a 10-fold increase in plasma resveratrol concentration ($P < 0.01$) when compared with a delivery vehicle consisting of polyethylene glycol and water.[11]

6.2.4 Enhanced Intestinal Permeability

Bioactives, such as peptides, have limited bioavailability due to inherently low intestinal membrane permeability.[62] Many large, hydrophilic bioactives are limited by poor transport through para- and trans-cellular routes due to their size or polarity.[63] In addition, the epithelial mucosa lining of intestinal cells have high affinity for cationic bioactives, which further reduces bioactivity.[64] Bioactive nanoencapsulation using liposomes or nanoparticles improves bioavailability of impermeable bioactives; this is an active area of research especially important in cancer research to increase drug permeability and retention in tumor cells.[65]

Numerous bioactive peptides, such as insulin, have low oral bioavailability (<1%) arising in part due to low epithelial permeability.[62–64,66,67] Currently, to overcome this barrier, most peptide bioactives are administered *via* parenteral routes such as injection, topical, and nasal.[66,68] The large molecular weight and high hydrophilicity of peptides with more than three amino acid residues typically have low mucus penetration, which reduces their ability to cross the intestinal membrane.[63] Numerous nanoencapsulation techniques have been shown to increase oral bioavailability of peptides by altering their interactions with the mucosa.[62,64–66] For example, encapsulation of *N*-trimethylated chitosan (TMC) with a disassociating outer layer of *N*-(2-hydroxypropyl) methacrylamide (HPMA), allows nanoparticles to efficiently permeate both the mucosa and cell membrane.[64] Encapsulation of insulin in HPMA-TMC nanoparticles increases permeability and ultimately *in vivo* bioavailability to 3.09% (TMC only) and 8.56% (HPMA-TMC) compared with free insulin, 1.36%.[64]

6.2.4.1 Enabling Tailored and/or Site-specific Release

Some nutraceuticals benefit from delayed- or sustained-release rather than immediate burst-release. Delayed-release begins after an initial lag time, allowing for passage through the harsh gastric conditions, before triggering bioactive release in a more favorable environment (*e.g.*, intestine). Sustained-release ensures a constant, often slower, bioactive release. In contrast, burst-release very quickly dissociates the active compound from the delivery

vehicle.[69] Controlled-release facilitates appropriate release to maintain circulating doses within the therapeutic window, which leads to less frequent dosing, reduction of high-dosage side-effects and over-dosage prevention.[59,70] Controlled-release is broadly used, as it provides bioactive protection through first-pass metabolism.[71]

Temporal controlled-release is time dependent, while distribution controlled-release is site specific.[59,72] Release profiles depend inherently on molecular weight, size, distribution morphology, bioactive carrier formulation, and externally on chemical environment (pH, ion-activated, hydrolysis-activated), biochemical environment (enzymatic release, small molecule activation) as well as physical forces (osmotic pressure, mechanical activation, *etc.*).[59] Other nutraceuticals may require targeting to specific cellular structures, cells, or organs for disease treatment. Targeting is done passively, coupling bioactives with macromolecules that are able to reach the site of interest, or actively (typically in conjugation with cell-specific ligands).[59,73] Encapsulation is one technique that is able to tailor site-specific delivery of bioactives, and is an extremely promising and active area of continued research.

Sustained-release is of particular interest for vitamin D and has been widely applied to bioactives from *Cruciferous* vegetables (*e.g.* indole-3-carbinol (I3C) and 3,30-diindolylmethane (DIM)).[70,74] Encapsulating vitamin D_3 with carboxylmethyl chitosan (CMCS), calcium, and zein decreased the vitamin D_3 burst-release to 40% at 1.5 hours compared with 60% for unencapsulated vitamin D_3.[70] Sustained vitamin D_3 release was much longer when encapsulated with zein and 20 : 1 CMCS:calcium (50% release in 6.5 hours) compared with the zein-alone nanoparticle (80% release in 6.5 hours).[70] Addition of I3C, DIM, and zein protein nanoparticles without a CMCS coating exhibited a burst-release of ~45–50% after 0.5 hours, whereas with the coating, the burst-release was reduced to ~40%.[74] The CMCS coating also prolonged the release profile of I3C and DIM (60% released at 6.5 hours) compared with 80% for zein particles.[74]

Soy protein isolate (SPI) and CMCS encapsulated bioactives have decreased release in gastric fluids and increased release in intestinal fluids.[75] CMCS-SPI nanoparticles had ~50% slower release rate than SPI particles, suggesting that CMCS provides a protective coating allowing intestinal targeting of bioactives.[75] Similarly, Volić *et al.* targeted intestinal release of essential oils using alginate and SPI spheres, which released ~40% of the essential oil in the simulated gastric phase, while 60% was released in the intestinal fluids after 2.5 hours.[76] Alginate-only spheres released ~70% of essential oil bioactives in gastric fluids and reached steady state in the intestinal fluids after only 75 minutes.[76]

6.2.4.2 *Flavor Masking*

An often-overlooked aspect of encapsulation for nutraceuticals is masking their pungent odors and flavors (*e.g.*, curcumin (earthy, spicy), polyphenols (astringent), and marine-sourced omega-3 fatty acids (fishy)).[77–80]

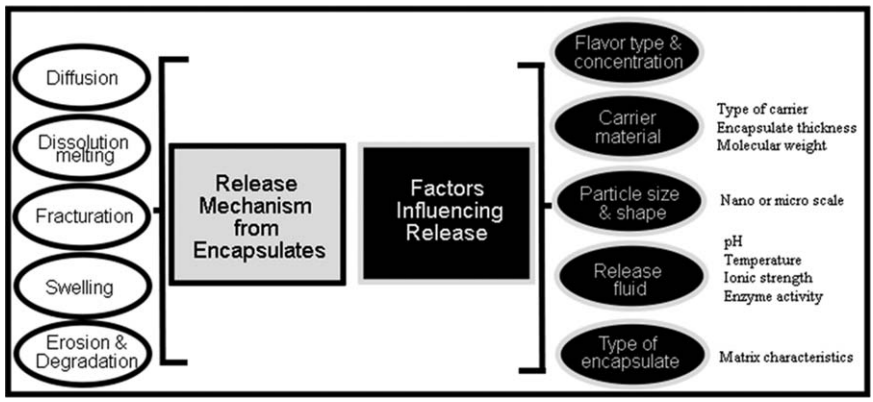

Figure 6.3 Factors that affect release of encapsulated bioactive flavors and their mechanism of release.
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Undesirable flavors and odors lead to poor consumer acceptability and provide a significant design challenge in developing functional foods.[79] Odors and flavors are commonly masked when encapsulated in the hydrophobic core of cyclodextrins, cyclic oligosaccharides consisting of α-(1,4)-linked D-glucopyranose units arranged in a ring formation commonly consisting of 6(α-), 7(β-), or 8(γ-) glucose units.[77,81] Cyclodextrin addition, even low concentrations, masks unpleasant flavors and aromas by non-covalently complexing with them in their inner core.[82] Flavor masking is dependent on the efficiency of the encapsulate to retain the bioactive, thus mechanisms that cause bioactive release may lead to off-flavors precipitation and decreased consumer acceptance (Figure 6.3). Cyclodextrins, at 0.5%, have been shown to mask the fishy taste of omega-3 fatty acids, while 1% was required to improve taste.[78] In citrus juices, cyclodextrin reduces free naringin concentration, an extremely bitter molecule of grapefruit juice, from 592 ppm to 316 ppm, while improving flavor preference by 22 points with a confidence interval of >99.9%.[83] Interestingly, the unpleasant bitterness of over-roasted coffee is also masked by addition of cyclodextrins (Hamilton and Heady 1970). Consumer acceptance of nutraceuticals or functional foods with off-flavors and -aromas is often limited by consumer preference and market advantages can be exploited by further developing encapsulation technologies.

6.3 Encapsulation Methods

Due to the molecular complexity and diversity of bioactives and desired release profiles, encapsulation technologies are individualistic in nature and include a plethora of delivery vehicles (Figure 6.4). However, our understanding of delivery design is rapidly expanding, and encapsulation methods

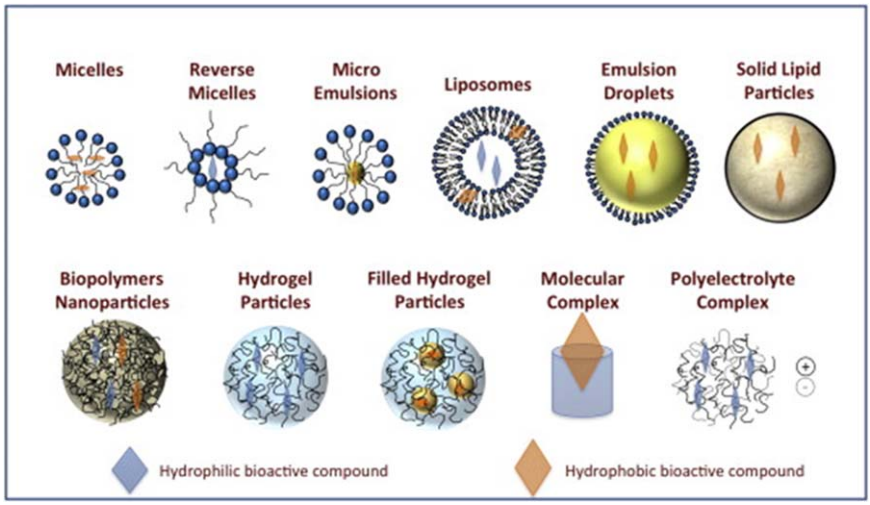

Figure 6.4 Colloidal delivery systems to encapsulate, protect and deliver bioactives. Reproduced from ref. 19 with permission from Elsevier, Copyright 2014.

are available at all stages of development, which can protect and improve nutraceutical bioaccessibility, absorption, and/or stability. Encapsulation technologies are broadly classified: (1) bottom-up approach (*e.g.*, spontaneous formation of particles due to molecular interactions); (2) top-down approach (*e.g.*, application of extensive external forces to disintegrate particles); and (3) a combination thereof. Selection of encapsulation technique depends on the physiochemical and molecular properties of the bioactive and the desired release profile.

6.3.1 Emulsification

Emulsification is not only important as an encapsulation method, but it is also used as a conditioning step prior to other encapsulation methods, such as spray or freeze drying. Emulsions are colloidal dispersions of two immiscible liquids, where one liquid is dispersed as a discontinuous phase forming discrete droplets suspended in a continuous phase. Amphiphilic emulsifiers reside at the interface between the two immiscible phases reducing the interfacial free energy and increasing the overall physical stability.[84] Formation of emulsions requires high-energy inputs, where the final droplet size depends on the magnitude of the energy input and Laplace pressure, which arises from the interfacial free energy and surface curvature of the discontinuous phase. Particle-size reduction, using the top-down approach, employs multiple devices including: high-shear mixers, high-pressure homogenizers (*e.g.*, ultrasonicators, or microfluidizers) each of which produce intense disruptive forces (*i.e.*, turbulent flow, shear, cavitation).[85]

Homogenization is commonly utilized to create emulsions with uniform particle sizes by passing a coarse emulsion through a narrow opening with high speed and pressure to facilitate the disruption of larger droplets into smaller particles. The fluid flow across the narrow opening initiates acceleration, causing laminar flow to become turbulent, which causes elongation of the droplets and eddies that reduce droplet size. The acceleration of fluid also leads to a significant pressure drop across the valve resulting in cavitation (*i.e.*, bubble formation). Microfluidizers pass two fast-moving liquids in opposite directions through microchannels and, upon collision, disruptive forces reduce droplet size with a narrow monomodal distribution.[86] Ultrasonication utilizes high-intensity ultrasonic sound waves as the disruptive forces to break down the droplets.

Unlike the top-down approach to manufacturing emulsions, low-energy, bottom-up approaches rely on phase inversion or spontaneous emulsification; however, these techniques produce less-stable emulsions and require higher concentrations of stabilizer.[19,87] Phase-inversion emulsification is triggered by changing temperature, known as the phase-inversion temperature (PIT) method, or by using a catastrophic phase change in the oil : water ratio, while maintaining surfactant concentration, known as the emulsion inversion point (EIP) method.[86] Using medium-chain triglyceride oil and Tween 40, 60 and 80, spontaneous emulsification encapsulated limonene into nanoemulsions with a particle diameter <40 nm.[87] Nanoencapsulation achieved using the solvent-displacement method involves spontaneous emulsification upon addition of an organic phase, with dissolved polymer and bioactive displaced into the aqueous external phase. Upon addition into the aqueous phase, the polymer precipitates out from the organic phase and forms encapsulates, while water-miscible organic solvent diffuses from the capsule into the aqueous phase. Encapsulation of β-carotene into poly(lactic acid) (PLA) and poly(lactic-*co*-glycolic acid) (PLGA) by the solvent-displacement method yielded spherical nanocapsules between 50 and 80 nm in diameter.[88]

6.3.2 Drying Methods

Drying techniques produce powdered encapsulates to improve stability, retention and shelf-life compared with their liquid counterparts.[85,89] Prior to spray drying, encapsulating material and bioactive are dispersed into an emulsion, the mixture is sprayed using an atomizer which ultimately defines droplet size. The carrier materials used are typically hydrophilic polysaccharides (*e.g.* maltodextrin, alginate, chitosan, carrageenan, *etc.*) and proteins (*e.g.* whey, soybean protein isolate, bovine serum albumin, *etc.*).[86] Spray drying is low cost, flexible and scalable while producing small, homogeneous, spherical particles; however, high temperatures result in a porous encapsulant structure that reduces shelf-life due to bioactive degradation *via* oxidation.[90] Spray drying, for the purpose of encapsulation, was done by de Paz *et al.* with β-carotene as a bioactive colorant in beverage

products.[91] Ethyl acetate-in-water emulsions, using modified *n*-octenyl succinate (OSA) starch and β-carotene produced an average droplet size of 500 nm, and spray drying increased particle size to 12 μm with an encapsulation efficiency of 65%.[91]

Spray chilling is similar to spray drying with the exception that a cooling chamber is used to crystallize the droplets. In addition, unlike spray drying, spray chilling requires a lipid-based bioactive carrier to facilitate crystallization.[86] After dispersing the bioactive in the oil, the sample is held below the lipid melting point.[89] Sillick and Gregson used spray chilling to encapsulate flavors using erythritol, an anhydrous sugar alcohol, as the carrier and found the loading capacity was 10%, while cinnamic aldehyde and limonene encapsulation efficiency was ~90%.[92]

Freeze drying, another common encapsulation technique, is utilized for thermally sensitive compounds. The four stages for freeze drying are: freezing, sublimation, desorption, and storage.[85] The mixture is first flash frozen, which immobilizes the continuous and discontinuous phases and then under vacuum sublimation occurs until the moisture content is $<5\%$.[86] The primary drawback is the long processing times and high energy requirements. Bejrapha and colleagues nanoencapsulated fish oil using both conventional (CFD) and vacuum-based (VFD) freeze drying.[93] Prior to drying, the encapsulation efficiency was ~87% and after freeze drying the efficiency of CFD and VFD was ~78% and 67%, respectively.[93] Both techniques successfully slowed oxidation of the omega-3 fatty acids in fish oil.[93]

Spray-freeze drying, a combination of freeze and spray drying technologies, atomizes the liquid sample before freezing and sublimation. This method produces higher-quality encapsulates (*i.e.*, improved storage stability and encapsulation efficiency) compared to individual spray or freeze drying.[86] Spray-freeze drying of docosahexaenoic acid (DHA) algae oil-in-water emulsions with Tween-40 prevents oxidation compared to spray drying and freeze drying, which were both ~33% (*versus* 14%).[94] Furthermore, the oxidative stability, as measured using the peroxidation value, of spray-freeze dried encapsulates improved compared to DHA-alone over 36 days at both ambient and refrigerated temperatures.[94]

6.3.3 Fluid Bed Coating

Fluid bed coating suspends solid particles in an upward stream of gas, which negates gravitational effects, causing particles to behave similarly to a fluid – hence the term fluidization.[95] Fluid bed coating applies various encapsulation wall materials onto bioactives suspended in the stream of gas. The encapsulate wall material is sprayed onto the fluidized bioactive particles and solvent is evaporated or solidified. Although this technique is similar to spray and freeze drying, it is not used extensively due to higher costs and the bioactive must be solid.[95] However, it allows for tailorable, narrow-distribution particle sizes, and allows for multiple layers of encapsulant.[96,97] For instance, emulsions with β-carotene, maltodextrin, and sodium

caseinate were spray dried before being further coated with hydroxypropyl cellulose using fluidized bed coating.[98] The particle size varied from 48.6 to 69.5 μm, with the smaller size occurring at higher operating temperature and feed rates.[98]

6.3.4 Coacervation

Coacervation employs liquid–liquid phase separation of colloid particles from a solution that forms nanocapsules ranging from 100 to 600 nm.[59,85] It requires three immiscible phases: a core material, coating material, and continuous liquid phase.[86] The hydrophilic encapsulate is deposited around the core droplets *via* interfacial adsorption to form a solid encapsulate, and cross-linking, heating or desolvation subsequently occur. Simple coacervation requires addition of a chemical (typically proteins) that compete with the colloidal solute for solubility causing precipitation and coacervate formation.[86] Complex coacervation uses oppositely charged hydrophobic colloids (*e.g.* gelatin and gum arabic) that form polyelectrolyte complexes and generate a driving force for phase separation, which is associated with their opposite charges.[69,90] However, coacervate stability is limited to a narrow pH and ionic strengths. Cross-linking is commonly employed to reinforce the encapsulating material, but regulations limit cross-linking reagents as most are not generally recognized as safe (GRAS), particularly glutaraldehyde which is often used to cross-link gelatin.[85,86,90] Coacervation to encapsulate 10% sweet orange oil, using soybean protein isolate, sucrose, and gum arabic, showed microencapsulation efficiencies of 95% *versus* 80% without the sucrose.[99] Coacervate yield is significantly reduced below pH 3.5 (pH 4 had maximum coacervate yield at ~57% compared to 35% below pH 3.5).[99] Curcumin, described earlier as $B(-)_{L,S}A(-)_{TJ,AT,ET}T(-)_{M,C}$, was encapsulated using complex coacervation with gum arabic and bovine serum albumin and had an encapsulation efficiency of ~92% at pH 3.7.[18,100]

6.3.5 Inclusion Complexation

Inclusion complexation encapsulates bioactives in the inner cavity of a host molecule.[69,86] Unfortunately, the food industry is limited by the number of GRAS host–complexes, which only includes cyclodextrins (α-, β-, γ-).[19] The bioactive non-covalently binds into the cavity of cyclodextrin. Inclusion complexation is commonly used to encapsulate volatiles and mask flavors/odors. Inclusion complexation of anti-microbial essential oils, *trans*-cinnamaldehyde and eugenol, with β-cyclodextrin improves solubility and masks the oils' flavor.[101] *trans*-Cinnamaldehyde and eugenol had encapsulation efficiencies of $84.70 \pm 0.02\%$ and $90.15 \pm 0.06\%$, respectively.[101] Vanillin is insoluble in water beyond 1% (w/w), but when encapsulated in β-cyclodextrin and freeze dried the complex had improved solubility.[102] Resveratrol, classified as $B(-)_S$, $A(-)_{ET}$, $T(-)_{M,CD}$ was encapsulated in β-cyclodextrin (β-CD) and hydroxypropyl-β-cyclodextrin (HP-CD) to increase

solubility; HP-CD had a higher linear phase-solubility compared to β-CD with rate constant (K_C) values at 298 K of 6778 M^{-1} and 1815 M^{-1}, respectively.[103] Furthermore, the antioxidant efficacy of resveratrol HP-CD and β-CD complexes were 6.66×10^3 and 1.66×10^3 $l^{-1}\,min^{-1}$ respectively, indicating that the complexes increase resveratrol solubility while preserving antioxidant capacity.[103]

6.3.6 Supercritical Fluid

Supercritical fluids (SCF), at pressures and temperatures above their critical points, behave as a gas and a liquid, resulting in high solubilization capacity as well as low surface tension and viscosity.[85,89] Supercritical fluids, typically CO_2, can either act as the solvent or antisolvent.[89] The solvent technique solubilizes the bioactive and encapsulation polymers in a SCF prior to evaporation. The antisolvent technique solubilizes the bioactive and carrier material in a solvent and, upon addition, the SCF preferentially dissolves the solvent causing the bioactive and carrier to precipitate resulting in encapsulated particles.[89] SCF encapsulation avoids the need for organic solvents and operates at low temperatures making it ideal for heat-sensitive compounds.[85]

6.3.7 Liposomes

Liposomes formed from amphipathic molecules, commonly phospholipids, self-assemble into single-walled or multiple-concentric bilayers with particle size ranging between 0.02 and 10 μm.[59] The aliphatic nature of the encapsulate enables loading of lipophilic bioactives in the liposome core within an aqueous environment.[89] Furthermore, the presence of both hydrophobic and hydrophilic regions facilitates entrapment of different active ingredients within a single delivery system.[89] Numerous well-established methods exist to form liposomes and include solvent evaporation, ultrasonication, and high-pressure homogenization. However, due to liposome instability, addition of cholesterol, glycerol, and/or ionic substances is required.[19] Vitamin E encapsulated in lyophilized proliposomes with a polyethylene glycol (PEG) coat enhances stability and encapsulation efficiency.[104] Furthermore, the cumulative release of vitamin E, examined using *in vitro* dialysis and simulated intestinal fluids, was significantly slower for encapsulated compared to free vitamin E.[104] Curcumin encapsulated with soy lecithin (SLP-WHITE) had loading efficiencies of 85.2% and mean particle diameter of 114 nm.[105] After 30 days, SLP-WHITE solutions showed no precipitation or phase separation, highlighting the potential for liposomes to be used as delivery vehicles for bioactives.[105]

6.3.8 Extrusion

Extrusion passes a carrier solution, including the bioactive, through a nozzle into a gelling solution that facilitates gelation *via* an ionic reaction and/or

the encapsulant is hardened *via* evaporation.[86,106] These encapsulants are less porous than spray-dried encapsulants and are larger (>500 μm) but require milder processing conditions (maximum processing conditions: 100 psi and 118 °C).[106] Quercetin, an extremely bitter flavonoid with strong antioxidant, anti-inflammatory, and anti-cancer properties, has a NuBACS classification of $B(-)_L$, $A(-)_{BP,ET}$, $T(-)_M$. To mask its bitter flavor, Khor *et al.* encapsulated quercetin using hot-melt extrusion with carnauba wax, shellac, or zein.[107] The microencapsulated powders were larger in size (10–100 μm), due to aggregation upon solidification, and pure quercetin was more bitter (measure output ~3 mV) than encapsulated quercetin (measure output ~0.5 mV) as analyzed using an electronic tongue (note: <1 mV is not be perceived as bitter).[107]

6.4 External Triggers for Site-specific Release

Another important consideration when designing encapsulation technologies for bioactives with targeted biological functions is the release location and time (Figure 6.5). Encapsulation enables bioactive retention; however, it must release the active along the human gastrointestinal tract to confer bioactivity. Diffusion, the primary release mechanism, releases the bioactive from the internal matrix to the surface of the capsule and then into the surrounding environment.[59,86,89] The encapsulate may need to swell when exposed to GI fluids before diffusion occurs.[86] Swelling increases particle pore size and release occurs when the pore size exceeds the molecular size of the bioactive. However, the release rate depends on confounding factors

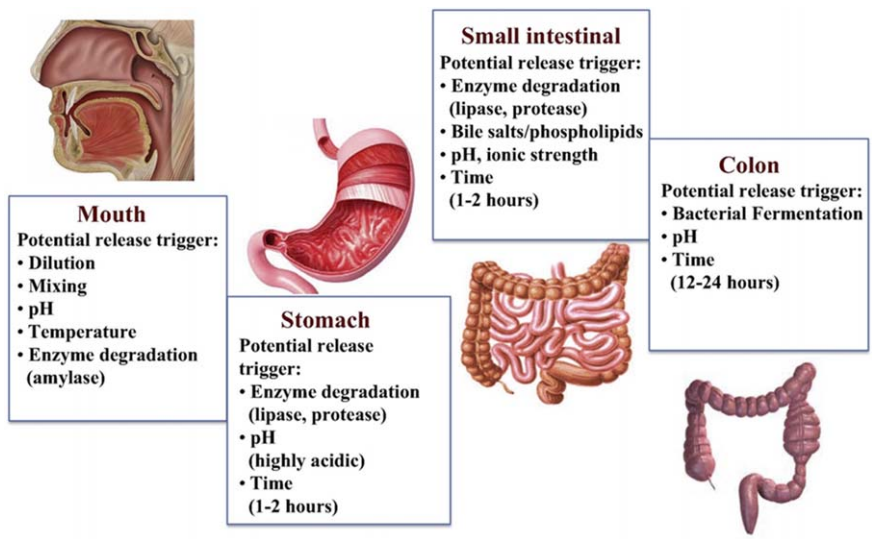

Figure 6.5 Potential bioactive release triggers along the gastrointestinal tract. Reproduced from ref. 108 with permission from Elsevier, Copyright 2015.

including solute properties (*e.g.*, molecular weight, polarity, pH), matrix properties (*e.g.*, polarity, rheology, physical state, interactions), particle characteristics (*e.g.*, size, shape, structure) and concentration gradient.[89] Although numerous triggers exist to initiate release (Figure 6.5), pH triggers release by altering the electrostatic non-covalent interactions between the encapsulate wall materials or the bioactives.[106] For example, calcium alginate hydrogels, formed through electrostatic cross-linking, can be triggered to release in specific pH environments. At low pH (*i.e.*, stomach), calcium alginate hydrogels shrink, decrease porosity, and retain the bioactive, while in higher pH environments (*i.e.*, duodenum, ileum, jejunum, colon) the encapsulate swells, decreases in porosity and releases the bioactive.[108] pH-dependent behavior is regularly used in designing targeted bioactive delivery systems.

Encapsulate coating material dissolution or melting is another commonly employed release-trigger mechanism for specific environments. Bioactive release rate is bottle-necked by the dissolution rate, which depends on the composition and encapsulant structure and environmental conditions (solvent, pH, ionic strength, temperature).[59,89] Release can also be triggered by encapsulate erosion, which may either be physical (*i.e.* due to temperature), chemical (*i.e.*, due to acids or bases), enzymatic (*i.e.*, *via* lipases, proteases and/or amylases activity) or a combination thereof. Release rate is highly influenced by particle composition and structure as well as the magnitude and duration of the exposure to the erosion factor.[59,89] For instance, the rate of lipid hydrolysis increases with increasing lipid particle surface area and thus smaller droplet size.[9] β-Carotene bioaccessibility in encapsulated filled-hydrogels (oil-in-water emulsion with whey protein isolate emulsifier gelled in rice starch) increased compared to the emulsion, as the hydrogel inhibited particle aggregation, facilitating rapid digestion of the encapsulate due to greater surface area for digestive enzymes to access.[109] Typically, release rate is controlled by altering diffusion, dissolution or erosion rates by modifying particle size and surface area.[59,89]

The location of bioactive release significantly impacts its bioavailability and efficacy.[19] Site-specific release therefore must consider the nature of the encapsulant wall-material, encapsulation technique and expected release mechanism.[97] Gastric release is often triggered by forming encapsulates containing highly digestible proteins that are enzymatically degraded by acidic proteases. Similarly, micronutrients that absorb in the small intestine are encapsulated in lipid-containing wall material that is enzymatically degraded by lipase. Colonic release uses encapsulates of dietary fiber.[19] For example, microparticles obtained by spray-chilling a double-layer emulsion (water/oil/water) containing fully-hydrogenated canola oil (internal wall material) and an external coating of sweet potato starch and isomalt only released $2.4 \pm 0.6\%$ of the core material after 3 hours in simulated gastric fluids; however, $59.8 \pm 0.2\%$ was released in simulated intestinal fluids after the same duration.[110] This delivery system shows potential for targeted intestinal delivery of water-soluble bioactives. For colon-targeted delivery on

the other hand, inulin is an excellent encapsulant, as it can survive the upper part of the GIT, is difficult to hydrolyze, and is not degraded until it encounters *Bifidobacterium* in the colon.[97]

6.5 Conclusions and Future Perspectives of Encapsulation Technologies

Consumer awareness of the relationship between diet and health increases market demand for healthier foods enabling growth within the nutraceutical and functional foods sectors. Although research has shown bioactives aid in preventing chronic diseases, numerous technological, physical, and biological barriers exist limiting them from being incorporated into formulations due to their instability, low-solubility, poor taste, and odor profiles. Encapsulation has emerged as a leading technology that allow bioactives to overcome these barriers. Furthermore, encapsulation technologies aid in controlling release, creating multifunctional or synergistic combinations by integrating multiple bioactives into a particle. Encapsulation technologies will facilitate science-based, personalized nutrition that allows consumers to choose a bioactive best suited for their individual needs.

It has been well-established that individuals respond differently to nutrition due to genetic variability, lifestyle, and environmental conditions.[111] Encapsulation technologies that improve bioaccessibility and bioavailability of bioactives may be desirable to subsets of the population. Targeted or tailored delivery of nutrients to certain cells due to encapsulation is useful in developing personalized nutrition to treat individuals with specific nutrient deficiencies or requirements.

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CHAPTER 7

Coffee and Solid Tumors

DONG HANG,[a,b] MINGYANG SONG[a,c] AND EDWARD L. GIOVANNUCCI*[a]

[a] Department of Nutrition, Harvard T.H. Chan School of Public Health, Boston, MA, USA; [b] Department of Epidemiology and Biostatistics, School of Public Health, Nanjing Medical University, Nanjing, China; [c] Department of Epidemiology, Harvard T.H. Chan School of Public Health, Boston, MA, USA
*Email: egiovann@hsph.harvard.edu

7.1 Cancer Incidence and Mortality

Cancer is a major public health problem worldwide. According to the World Cancer Report (http://www.who.int/mediacentre/factsheets/fs297/en/), cancer is the second leading cause of death and was responsible for an estimated total of 9.6 million deaths in 2018. Globally, about 1 in 6 deaths are due to cancer. The most common cancers include lung, breast, colorectal, prostate, and skin, with the most common causes of cancer death due to solid tumors of the lung, colorectal, stomach, liver, and breast. In the United States, it was estimated that 1 762 450 new cancer cases and 606 880 cancer deaths would occur in 2019.[1] Approximately 38.4% of US men and women will be diagnosed with cancer at some point during their lifetime and one in four deaths will be due to cancer. In 2017, estimated US expenditure for cancer care was $147 billion and this cost is likely to increase as the population ages and the number of cancer survivors increases (https://www.cancer.gov/aboutcancer/understanding/statistics).

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7.2 Cancer Etiology

Cancer is a complex disease caused by both internal factors (such as germline mutations, hormones, and immune dysfunction) and environmental/acquired factors (such as tobacco, diet, radiation, and infectious organisms). Accumulating evidence supports that cancer development is a multistep process characterized by a series of molecular changes at genetic and epigenetic levels, which drive the transformation of a normal cell into a cancer cell. The process typically takes decades to complete. In 2011, Hanahan and Weinberg proposed that the malignant transformation and progression requires ten hallmarks of cellular physiological rearrangements, including (1) sustaining proliferative signaling, (2) evading growth suppressors, (3) resisting cell death, (4) enabling replicative immortality, (5) inducing angiogenesis, (6) activating invasion and metastasis, (7) reprogramming energy metabolism, (8) evading immune destruction, and the two enabling characteristics that include (9) genome instability that provides mutations and (10) inflammation.[2] This framework is helpful in identifying anti-tumor agents by examining their effects on each of the hallmarks.

7.3 Coffee and Anti-cancer Compounds

Coffee is one of most widely consumed beverages globally, with an estimated consumption of more than 2.25 billion cups per day.[3] According to the National Coffee Association's report in 2017, 62% of US adults drink coffee daily.[4] Besides caffeine, coffee contains over 1000 bioactive compounds, including trigonelline, polyphenols (*e.g.*, chlorogenic acids), lipids in the form of diterpenes (*e.g.*, cafestol and kahweol), and melanoidins. These compounds may influence carcinogenesis by acting upon different pathways that are important for cancer development and progression. Some major effects of the specific compounds are summarized in Table 7.1 and in the following sections.

7.3.1 Caffeine

Caffeine (1,3,7-trimethylxanthine), a purine alkaloid, is mainly known for its ability to increase blood pressure and psychostimulatory and diuretic properties.[5] Coffee roasting, the process that transforms green coffee beans into black coffee beans, can decrease the concentrations of caffeine. Moreover, caffeine can be removed from coffee to provide decaffeinated coffee. In most populations, coffee and tea are the major sources of caffeine.

Studies have shown that caffeine is a potent anti-oxidant and radioprotector against oxidative DNA damage through quenching the production of hydroxyl radicals.[6] Caffeine also has the ability to reduce the expression of pro-inflammatory genes and inhibit the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in a range of cells and organisms, thereby exerting anti-inflammatory activities.[7] In addition, caffeine

Table 7.1 Primary bioactive compounds of coffee and their anti-cancer mechanisms.

	Bioactive compounds				
Cancer hallmark	Caffeine	Trigonelline	Chlorogenic acids	Cafestol/ kahweol	Melanoidins
Genome instability	↓[6]	↓[9]	↓[13]	↓[16]	↓[19]
Inflammation	↓[7]		↓[15]	↓[17]	↓[23]
Proliferative signaling	↓[8]	↓[10]	↓[14]		↓[21]
Evading growth suppressors	↓[8]		↓[14]		
Resisting cell death	↓[8]		↓[14]		
Angiogenesis				↓[17]	
Invasion and metastasis	↓[96]	↓[10]	↓[97]	↓[98]	↓[99]
Reprogramming energy metabolism		↓[9]	↓[13]	↓[16]	↓[20]

can suppress epidermal growth factor-caused cell malignant transformation and induce cell cycle arrest and apoptotic response in cancer cell lines.[8]

7.3.2 Trigonelline

Trigonelline is a plant alkaloid derivate of vitamin B6 contained in coffee beans. It is also one of the main components that contribute to the characteristic bitterness in coffee. Evidence from animal studies has shown that trigonelline decreases malonaldehyde and nitric oxide concentrations and increases the activity of superoxide dismutase, catalase, glutathione, and inducible nitric oxide synthase in the pancreas.[9] These data suggest that trigonelline may exert antioxidant effects by upregulating anti-oxidant enzyme activities and decreasing lipid peroxidation. In fact, trigonelline is the first chemically identified hormone that promotes cell arrest in animal tissues.[10] In cancer cells, trigonelline and niacin treatment can significantly reduce levels of reactive oxygen species and inhibit cancer invasion.[11]

7.3.3 Phenols and Polyphenols

Coffee is also a rich source of phenols and polyphenols, a heterogeneous group of secondary metabolites synthesized by plants to defend against pathogens and environmental risk factors. The most abundant group of phenolic compounds in coffee consists of approximately 45 different compounds known as chlorogenic acids, which are formed by an esterified quinic acid moiety and one or several hydroxycinnamic acid moieties, such as caffeic, ferulic, *p*-coumaric, and vanillic acid.[12] Polyphenols have strong anti-oxidant properties and can inhibit oxidative stress and oxidative DNA damage.[13] *In vitro* evidence also supports a protective role of polyphenols in

carcinogenesis by exerting anti-proliferative effects and inducing cell cycle arrest and/or apoptosis.[14] Moreover, chlorogenic acids have been shown to suppress the production of inflammatory mediators by inhibiting protein tyrosine phosphatase 1B, reducing the expression of proinflammatory cytokine genes, and modulating NF-κB activation.[15]

7.3.4 Lipids

Coffee contains lipids, which are mainly found in the form of two diterpene alcohols—cafestol and kahweol. These molecules are sensitive to the roasting process and can be largely removed from coffee when it is brewed using a paper filter. Relatively high levels of cafestol and kahweol are present in unfiltered coffee such as espresso, French press, boiled coffees or Turkish/Greek coffee. Experimental studies have shown that cafestol and kahweol can act as anti-genotoxic compounds that prevent DNA damage from several genotoxic carcinogens and inhibit the expression and/or activity of cytochromes P450 involved in carcinogen activation.[16] They may also increase the activity of enzymes that are involved in detoxification of DNA-reactive metabolites, such as glucuronosyltransferase and glutathione *S*-transferase, and influence the DNA-repair machinery by activating DNA-repair enzymes.[16] Furthermore, kaweol and cafestol have inhibitory effects on inflammation and angiogenesis.[17]

7.3.5 Melanoidins

Melanoidins are a heterogeneous group of complex polymeric macromolecules that are predominantly responsible for the dark brown color of coffee. Melanoidins are produced by the Maillard reaction during the roasting and heating processes from polysaccharides (arabinogalactans and galactomannans), free amino groups from amino acids or proteins, and phenolic compounds, mainly chlorogenic acids.[18] Existing evidence supports the biological activities of melanoidins against carcinogenesis. For example, melanoidin possesses a high anti-oxidant activity and protects cells against lipid peroxidation and DNA oxidative damage.[19,20] *In vitro*, melanoidins reduce cancer cell proliferation by regulation of cell cycle proteins.[21,22] Moreover, melanoidins may reduce concentrations of proinflammatory cytokines such as tumor necrosis factor-α and interferon-γ and increase of anti-inflammatory cytokines such as interleukin-4.[23]

7.4 Coffee Intake and Cancer Risk

In 1991, the International Agency for Research on Cancer (IARC) classified coffee as a possible cause of cancer based on studies that linked coffee consumption to increased risk of bladder cancer.[24] Since then, the associations between coffee intake and cancer risk have been widely investigated. IARC now states that there is no conclusive evidence for a carcinogenic effect

of coffee intake.[25] Coffee is a source of dietary acrylamide, a carcinogen, but the evidence that dietary acrylamide is associated with cancer risk in levels typically consumed is not strong.[26] Coffee consumers are more likely to be smokers and some of the initial studies had not adequately accounted for the confounding effects of smoking when examining the association between coffee and cancer. On the other hand, accumulating evidence indicates the benefit of coffee for cardiometabolic health and reducing cancer risk. Given these data, the 2015 US Dietary Guidelines Advisory Committee recommended to incorporate moderate coffee consumption into a healthy dietary pattern.[27]

7.4.1 Endometrial Cancer

A protective association between coffee intake and endometrial cancer risk was first reported in a Norwegian prospective study in 1986.[28] Subsequently, many case-control and cohort studies have revisited the hypothesis, with the findings summarized in several meta-analyses, all of which showed an inverse association between coffee consumption and endometrial cancer risk.[29–34] The most recent meta-analysis reported the results of 12 cohort studies with 11 663 endometrial cancer cases and 8 case-control studies with 2746 cases.[34] Comparing the highest to the lowest category of coffee intake, the summary relative risk (RR) was 0.78 [95% confidence interval (CI): 0.71–0.85] in cohort studies and 0.63 (95% CI: 0.53–0.76) in case-control studies. The association was similar for caffeinated coffee and decaffeinated coffee. In dose–response analysis, one-cup increment per day was associated with a 3% (95% CI: 2–4%) risk reduction of endometrial cancer in cohort studies and 12% (95% CI: 5–18%) in case-control studies. Based on these data, the report from the World Cancer Research Fund/American Institute for Cancer Research concludes that coffee is a protective factor against endometrial cancer (https://www.wcrf.org/sites/default/files/Endometrial-cancer-report.pdf).

Besides the above-mentioned anti-cancer properties of coffee ingredients, some other mechanisms have been proposed to explain the association between coffee intake and endometrial cancer. For example, caffeine and coffee intake have been linked to increased levels of sex hormone-binding globulin (SHBG) and reduced levels of estrogen.[35,36] SHBG is the major carrier for estrogen and androgen, thus down-regulating the circulating levels of free hormones. Higher levels of SHBG and lower levels of estrone and estradiol have been associated with reduced risk of hormone-related cancers,[37] including endometrial cancer. In addition, coffee intake may lower levels of circulating estrogens by inhibition of the enzyme converting androgens into estrogens, *i.e.*, cytochrome P450, family 19 or aromatase.[36] Lower levels of estrogens may play a protective role in endometrial cancer by down-regulation of endometrial proliferation.[38] Evidence also indicates that coffee consumption decreases the risk of diabetes,[39] an important risk factor for various types of cancer, including endometrial cancer.

7.4.2 Liver and Biliary Tract Cancers

In the Liver Cancer Pooling Project, a consortium of US-based cohort studies, an individual-based pooled analysis of 9 cohorts with 1 212 893 participants showed that higher coffee consumption was associated with lower risk of hepatocellular carcinoma (RR = 0.73, 95% CI: 0.53–0.99 for >3 cups per day *versus* non-drinker).[40] According to a recent dose–response meta-analysis of 18 cohort and 8 case-control studies, each two cups per day of coffee intake was associated with a 35% lower risk of hepatocellular carcinoma (95% CI: 28–41%).[41] The inverse association was weaker for cohort (RR = 0.71, 95% CI: 0.65–0.77) than case-control studies (RR = 0.53, 95% CI: 0.41–0.69). Both caffeinated and decaffeinated coffee showed an inverse association with hepatocellular carcinoma risk (RR = 0.73, 95% CI: 0.63–0.85 and RR = 0.86, 95% CI: 0.74–1.00, respectively). Similar results were reported in a more recent Japanese cohort study among 172 liver cancer patients.[42] Thus, as the World Cancer Research Fund and American Institute for Cancer Research state (https://www.wcrf.org/dietandcancer/liver-cancer), there is strong evidence supporting a protective effect of coffee intake on liver cancer.

In terms of mechanisms, several studies have found a favorable effect of coffee consumption on liver function. For example, caffeine has been shown to counteract the hepatic fibrinogenesis pathway by downregulating the production of connective tissue growth factor induced by transforming growth factor-β1, by upregulating peroxisome proliferator activated receptor-γ expression and inhibiting the synthesis of focal adhesion kinase and actin.[43] In addition, coffee intake is associated with reduced serum concentrations of γ-glutamyltransferase, aspartate aminotransferase, and alanine aminotransferase, as well as lower risk of severe steatohepatitis in patients with nonalcoholic fatty liver disease and liver fibrosis, both of which are chronic alterations preceding liver cancer.[44] Furthermore, some studies have reported an inverse association between coffee consumption and levels of systemic immune and inflammatory markers,[45–47] suggesting that coffee consumption may alleviate the inflammation processes that characterize nonalcoholic fatty liver disease, steatohepatitis, and liver fibrosis.

Epidemiologic evidence has also shown an inverse association between coffee consumption and risk of gallstones, a main risk factor for gallbladder cancer.[48] According to a recent prospective cohort study of 72 680 Swedish adults and 74 incident gallbladder cancer cases, the multivariable RR was 0.76 (95% CI: 0.41–1.41) for 2 cups per day, 0.50 (95% CI: 0.24–1.06) for 3 cups per day, and 0.41 (95% CI = 0.20–0.83) for ≥4 cups per day, compared with consumption of ≤1 cup of coffee per day.[49] The results were in line with the findings of previous case-control studies.[50,51] Mechanistically, coffee consumption can stimulate cholecystokinin release, enhance gallbladder contraction, and decrease cholesterol crystallization in bile.[52,53] Therefore, it is possible that coffee consumption has a protective effect on gallbladder cancer.

7.4.3 Oral and Pharyngeal Cancers

A meta-analysis of 11 case-control and 4 cohort studies that included 5021 cases of oral cancer indicated that the summary odds ratio (OR) comparing the highest to the lowest category of coffee consumption was 0.63 (95% CI: 0.52–0.75).[54] The ORs were 0.60 (95% CI: 0.49–0.74) in case-control studies and 0.66 (95% CI: 0.45–0.98) in cohort studies. A recent meta-analysis of 13 case-control and 4 cohort studies reported a summary OR of 0.69 (95% CI: 0.57–0.84) comparing high to low category of coffee consumption for oral and pharyngeal cancers combined, an OR of 0.82 (95% CI: 0.58–1.16) for oral cancer, and an OR of 0.72 (95% CI: 0.54–0.95) for pharyngeal cancer.[55] When stratified by study design, the ORs were 0.67 (95% CI: 0.53–0.84) in case-control studies and 0.86 (95% CI: 0.69–1.08) in cohort studies. Therefore, cumulating epidemiologic evidence suggests that coffee consumption is associated with a lower risk of cancers of the oral cavity and pharynx.

It is possible that the anti-oxidant activity of coffee may prevent oxidative damage induced by smoking and alcohol, the main risk factors for oral and pharyngeal cancer.[56] Further studies are needed to elucidate the mechanisms underlying the benefit of coffee constituents for oral and pharyngeal cancers.

7.4.4 Prostate Cancer

Four meta-analyses have consistently reported an inverse association between coffee consumption and prostate cancer risk.[57–60] In the most recent meta-analyses, including 13 cohort studies with 34 105 cases, the summary RR for the highest *versus* lowest coffee intake was 0.90 (95% CI: 0.85–0.95).[60] The dose–response analysis showed a lower cancer risk by 2.5% (95% CI: 0.5–4.3%) for every 2 cups per day increment. In the stratified analysis, the RRs were 0.89 (95% CI: 0.83–0.96) for non-advanced, 0.82 (95% CI: 0.61–1.10) for advanced, and 0.76 (95% CI: 0.55–1.06) for fatal prostate cancer. Therefore, current evidence suggests that coffee consumption may be associated with a lower risk of prostate cancer.

Coffee is a major source of anti-oxidants that have potential anti-proliferative and anti-metastatic activities on prostate cancer cells.[61] Moreover, coffee has been linked to higher levels of testosterone, SHBG, and adiponectin,[36,62,63] all of which are associated with decreased risk of prostate cancer. Therefore, biological evidence supports that coffee may inhibit prostate carcinogenesis through various pathways.

7.4.5 Skin Cancer

Several studies have evaluated the association between coffee consumption and melanoma risk, but the results are mixed. In a meta-analyses including 2 case-control and 5 cohort studies, caffeinated coffee, but not decaffeinated coffee, was inversely associated with melanoma risk.[64] The summary RR was

0.81 (95% CI: 0.68–0.97) comparing the highest to lowest category of caffeinated coffee. According to a dose–response meta-analysis of 7 cohort studies with 9211 melanoma cases, increasing coffee consumption by one-cup per day was associated with a 3% (95% CI: 1–5%) reduction in melanoma risk.[65]

Some studies have also investigated the association between coffee intake and non-melanoma skin cancer (NMSC). According to a meta-analysis of 13 studies comprising 37 627 patients, intake of caffeinated coffee was inversely associated with NMSC risk (OR = 0.82 for the highest *versus* lowest intake category, 95% CI: 0.75–0.89).[66] A similar association was observed for caffeine intake (OR = 0.86 for the highest *versus* lowest intake category, 95% CI: 0.80–0.91). In subgroup analysis, the inverse associations were only detected for basal cell cancer. There was no association between decaffeinated coffee and NMSC risk. Therefore, epidemiologic evidence supports that caffeinated coffee intake was associated with lower risk of melanoma and basal cell cancer.

Besides the aforementioned anti-carcinogenic actions, caffeine may also have specific effects on skin cells. Experimental studies suggest that caffeine could inhibit UV-induced formation of thymine dimers and sunburn lesions in the epidermis of mice, enhance elimination of damaged pre-cancerous cells, induce apoptosis of tumor cells, and reduce the inflammation of skin cells.[67,68] Therefore, higher coffee or caffeine intake may have preventative effects against skin cancer.

7.4.6 Breast Cancer

A number of epidemiologic studies have investigated the association between coffee consumption and breast cancer risk, but yielded inconsistent results. An earlier meta-analysis reported a significant inverse association between coffee intake and estrogen receptor (ER)-negative breast cancer.[69] In another meta-analysis that pooled the data from 20 case-control and 17 cohort studies, a significant inverse association between coffee intake and breast cancer risk was observed among postmenopausal women and BRCA1 mutation carriers.[70] Similarly, according to an up-to-date meta-analysis including 21 cohort studies, an inverse relationship was observed when the analysis was restricted to postmenopausal women.[71] Four cups per day of coffee intake was associated with a 10% (95% CI: 1–18%) reduction in postmenopausal breast cancer risk. Therefore, coffee consumption might have a protective role in breast cancer among specific subgroups of women (*i.e.*, based on receptor and menopausal status).

Coffee might affect breast carcinogenesis through its effects on sex hormones and metabolic pathways. For example, coffee intake has been positively associated with SHBG and adiponectin,[36,63] and inversely with estradiol and leptin.[35,36,72] High levels of SHBG may reduce circulating estrogen levels. In addition, coffee might contribute to higher levels of plasma enterolactone, a phytoestrogen inversely associated with ER-negative breast cancer risk.[73]

7.4.7 Colorectal Cancer

Based on data from 25 case-control studies, a meta-analysis reported an inverse association with colon cancer (OR = 0.79, 95% CI: 0.67–0.95), but not rectal cancer (OR = 0.95, 95% CI: 0.79–1.15) comparing the highest to the lowest category of coffee consumption.[74] Similarly, in a subsequent meta-analysis of 19 cohort studies including 22 629 patients, an inverse but weak association was observed for colon cancer (RR = 0.93 for 4 cups per day *versus* nondrinker, 95% CI: 0.88–0.99).[75] However, no association was observed in a large pooling project of 13 prospective cohort studies.[76] Taken together, these data suggest a modest, if any, protective effect of coffee consumption on colon cancer.

Possible mechanisms for the benefit of coffee on the colon include reduced synthesis and secretion of bile acids, potential promoters of colon carcinogenesis,[77] and increased rectosigmoid motility that reduces exposure to carcinogens for epithelial cells.[78] An inverse association between coffee intake and circulating C-peptide (a stable marker of insulin secretion) also supports the role of coffee consumption in metabolic modulation,[79] thereby potentially lowering colon cancer risk.

7.4.8 Bladder Cancer

Numerous studies have investigated the association between coffee consumption and bladder cancer, but the results are mixed. In a meta-analysis of 34 case-control and 6 cohort studies with 16 172 cases, an increased risk of bladder cancer was observed for the highest to lowest intake category in men (RR = 1.31, 95% CI: 1.08–1.59), but not in women (RR = 1.30, 95% CI: 0.87–1.96). Of note, the separate analysis by study design showed that the association with increased risk was restricted to case-control studies. A recent Japanese cohort study with 274 incidents of bladder cancer reported an inverse association between coffee intake and bladder cancer, after adjusting for various confounding variables including smoking.[80] Further investigation with sufficient confounding adjustment is required to further evaluate the association between coffee consumption and risk of bladder cancer.

7.4.9 Lung Cancer

Many epidemiologic studies have evaluated the association between coffee consumption and risk of lung cancer, but the results are inconsistent. An important aspect to consider is the potential confounding effect by tobacco smoking, which has been positively associated with coffee consumption in various populations.[81] Although several studies have attempted to control for the confounding effect of tobacco smoking *via* statistical adjustment, residual confounding is difficult to rule out completely because the magnitude of the association between smoking and lung cancer is so strong. In a

meta-analysis of 8 prospective cohort and 13 case-control studies, a positive association of borderline statistical significance was observed between coffee drinking and lung cancer risk among nearly 20 000 cases of lung cancer (RR = 1.09, 95% CI: 1.00–1.19); however, no association was found in nonsmokers (OR = 0.92, 95% CI: 0.75–1.10).[82] Thus, coffee intake does not appear to be a risk factor for lung cancer once the confounding effect by smoking is controlled for.

7.4.10 Pancreatic Cancer

In 1981, a highly prominent case-control study reported a positive association between coffee intake and pancreatic cancer.[83] However, in retrospect, the control group was likely to be biased in excluding coffee drinkers, which may have biased the overall result. Subsequently, many studies have examined the relationship and yielded inconsistent results. A meta-analysis of 20 cohort studies reported an overall RR of 0.75 (95% CI: 0.63–0.86) comparing the highest to lowest category of coffee consumption.[84] In contrast, another meta-analysis of 28 studies with smoking adjustment reported no association between coffee consumption and pancreatic cancer (RR = 1.03 for the increment of one cup per day, 95% CI: 0.99–1.06).[85] Recently, the UK prospective Million Women Study, which included 962 pancreatic cancer cases in never-smokers, reported a RR of 0.87 (95% CI: 0.64–1.18) comparing $\geq$5 cups of coffee daily to non-drinkers.[86] A meta-analysis combining this cohort with three other prospective studies also found no statistically significant association between coffee consumption and pancreatic cancer risk among never smokers. Thus, existing data indicate that, after adjusting for smoking, there is no association between coffee intake and pancreatic cancer risk.

7.4.11 Ovarian Cancer

In a large cohort study, the European Prospective Investigation into Cancer and Nutrition, which included 1244 ovarian cancer patients, found no association between coffee consumption and risk of ovarian cancer comparing the top quintile of intake to nondrinking (RR = 1.05, 95% CI: 0.75–1.46).[87] The null result was confirmed by two subsequent meta-analyses including 7 and 8 cohort studies, respectively.[87,88] Therefore, epidemiologic evidence does not support an association between coffee and risk of ovarian cancer.

7.4.12 Others

Limited data suggest a lack of association of coffee intake with risk of other types of cancer, including those of the esophagus, small intestine, kidney, brain, thyroid, and soft tissue sarcoma.[89]

7.5 Coffee Intake and Cancer Mortality

The majority of early studies reported null association between coffee consumption and total cancer mortality (*i.e.*, the number of cancer deaths in a specified population initially free of cancer per year).[90] However, these studies may be prone to confounding effects by smoking. In a recent dose–response meta-analysis of 31 cohort studies including 40 991 cancer deaths, a linear association with cancer mortality was observed for one cup per day increase (RR = 0.98, 95% CI: 0.96–1.00) among non-smokers.[91] For site-specific mortality, a large cohort study including 118 738 cancer-related deaths reported that, among nonsmokers, two cups per day increase in coffee consumption was associated with lower risk of mortality of colorectal cancer (RR = 0.97, 95% CI: 0.95–0.99), liver cancer (RR = 0.92, 95% CI: 0.88–0.96), and breast cancer (RR = 0.97, 95% CI: 0.94–0.99). A nonlinear inverse association was also observed for head and neck cancer starting at 2–3 cups per day (RR = 0.72, 95% CI: 0.55–0.95).[92] In addition, two independent studies reported an inverse association between coffee consumption after diagnosis and colorectal cancer prognosis.[93,94] Compared with nondrinkers, colorectal cancer patients who consumed at least 4 cups of coffee per day had a 52% (95% CI: 17–72%) lower risk of colorectal cancer-specific death.[93]

Cancer mortality in a population is influenced both by cancer incidence rates and survival rates from these cancers. Beyond plausible mechanisms regarding coffee and the occurrence of cancer, an effect of coffee on cancer survival has been supported by experimental evidence. Caffeine has been shown to enhance the radiosensitivity of tumor cells by inducing apoptosis and inhibiting metastasis by regulating the epithelial–mesenchymal transition.[95,96] Chlorogenic acid may inhibit colon cancer metastasis by targeting mitogen-activated protein kinases and T-cell-originated protein kinase.[97] In addition, kahweol has been reported to inhibit metastasis by disrupting signal transducer and activator of transcription-3 (STAT3)-mediated transcription of the pro-metastatic genes (*e.g.*, matrix metalloproteinase (MMP) and vascular endothelial growth factor (VEGF)).[98] Melanoidins may also have the potential to inhibit matrix metalloproteases, important enzymes for metastasis.[99]

7.6 Conclusions

Substantial evidence indicates that higher coffee consumption is associated with lower risk of endometrial and liver cancers. Limited data suggest a potential benefit for cancers of the gallbladder, oral cavity and pharynx, prostate, skin, and breast. There is no strong evidence of an increased risk of any cancer associated with coffee consumption. Despite the limitations of observational studies (*e.g.*, residual confounding), the robustness of the findings across different study populations and the consistency of mechanistic evidence indicate a potential biological benefit of coffee consumption on carcinogenesis. In many populations, higher coffee intake is

associated with higher likelihood of cigarette smoking and alcohol consumption, among the strongest risk factors for cancer. Thus, one would generally expect confounding from these factors to result in an apparent positive, rather than inverse, association between coffee intake and cancer. Among individual food or beverage items, coffee has among the best evidence for a cancer prevention effect across multiple cancers from an epidemiologic perspective. Part of the reason may be that coffee is widely consumed, with a wide range of intake, making it amenable to study in multiple populations. Coffee is a source of many bioactive compounds, many of which have plausible anti-cancer properties. Further investigations are needed to better characterize the minimum dose of coffee consumption needed for its chemopreventive effects and the impact of modifying brewing methods on coffee bioactivity. Additional work is needed to examine and better define the underlying mechanisms of this bioactivity, identify the specific anti-cancer ingredients, and assess the potential role of coffee consumption in adjuvant cancer therapy.

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CHAPTER 8

Nutraceuticals in Hematological Malignancies

MATTHEW TCHENG,[†] ALESSIA ROMA[†] AND PAUL A. SPAGNUOLO*

Department of Food Science, University of Guelph, 50 Stone Rd., Guelph, Ontario, Canada N1G2W1
*Email: paul.spagnuolo@uoguelph.ca

8.1 Introduction

Hematological malignancies (HMs) are a set of blood, bone marrow and lymphatic tissue disorders with varying disease risk, etiology and prognoses.[1] Lymphoid malignancies occur in 15 and 23 of 100 000 females and males, respectively, while myeloid malignancies occur in 3 and 4 of 100 000 females and males, respectively.[2] Given the heterogeneity among HMs, diverse therapeutics are utilized to achieve remission. Several highly successful, frontline chemotherapeutics are structural derivatives of nutraceuticals. For example, all-*trans* retinoic acid (ATRA) is a vitamin A-derivative used for the treatment of acute promyelocytic leukemia (APML), a subtype of acute myeloid leukemia (AML) defined by the promyelocytic leukemia-retinoic acid receptor alpha (PML-RARA) fusion gene.[3] It promotes the differentiation of leukemic promyelocytes into mature granulocytes and, when used as a single agent, induces complete remission (CR) in 85% of patients.[4] Prior to ATRA, APML was a fatal disease;[5] however, recent trials have shown that

[†]Authors contributed equally to this work.

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drug combination therapies result in 100% of patients attaining CR at 50 months.[6] The success of ATRA for the treatment of APML highlights the potential utility of nutraceuticals and supports their use as an investigative tool for the discovery of additional therapeutics.

A growing population of cancer patients use alternative medicines and/or nutraceuticals. In Europe, it was reported that 26.5% of HM patients use complimentary or alternative medicine. Of concern, nearly 50% of cancer patients using nutraceuticals do not disclose this information to their primary physicians.[7,8] Although potentially beneficial, nutraceuticals can negatively interact with treatment regimens, posing a significant health risk.[9] Given this, nutraceuticals and their potential risks and benefits to HM patients must be defined. This review summarizes clinical studies (Table 8.1) that investigate the anti-cancer effects of nutraceuticals and aims to determine their ability to improve well-being and prevent disease progression in HM patients.

8.2 Hematological Malignancies

Hematological malignancies can be subdivided into three broad categories: (1) myeloid *versus* lymphoid, (2) chronic *versus* acute and (3) Hodgkin's *versus* non-Hodgkin's lymphoma. Within these, additional subtypes (Figure 8.1) categorized based on histopathological and cytogenetic characteristics are discussed below.

8.2.1 Lymphoid Malignancies

8.2.1.1 Chronic Lymphocytic Leukemia (CLL)

CLL is characterized by the abnormal accumulation and proliferation of lymphoid B-cells[10] and is the most prevalent leukemia in Western populations. Incidence and disease aggressiveness increases with patient age.[11] While manifesting primarily in the peripheral blood, malignant lymphocytes may spread to the lymph nodes and spleen.[10] With its slow progression, chemotherapy is typically not initiated until there is a substantial increase in absolute lymphocyte counts (ALC) or lymphadenopathy (*i.e.*, the enlargement of the lymph nodes).[11] Chemotherapy typically involves chlorambucil, an alkylating agent, which is administered to achieve initial remission. Upon relapse, a combination of **f**ludarabine, **c**yclophosphamide, **r**ituximab (FCR) has been shown to improve event-free and overall survival in patients.[11] Chlorambucil and FCR regimens rarely result in permanent remission and may cause cytopenia and myelodysplasia, which makes prolonged regimens impractical.[11]

8.2.1.2 Lymphomas

Lymphomas refer to a diverse spectrum of HMs that involve dysregulated proliferation and differentiation of lymphocytes in the peripheral blood and lymphoid tissues.[12] Approximately 90% of lymphomas are classified as

Table 8.1 Clinical studies involving the use of nutraceuticals to treat hematological malignancies.

Nutraceutical intervention	Title of clinical study	Disease	Trial identifier or PMID	Summarized results
Turmeric+imatinib	Effect of imatinib therapy with and without turmeric powder on nitric oxide levels in chronic myeloid leukemia.	CML	PMID: 21844132	Combination therapy reduced nitric oxide more than imatinib alone.
Curcumin	The Potential Role of Curcumin in Patients with Monoclonal Gammopathy of Undefined Significance—Its Effect on Paraproteinemia and the Urinary N-Telopeptide of Type I Collagen Bone Turnover Marker	MGUS	PMID: 19737963	Curcumin slowed progression from MGUS to multiple myeloma (MM).
Curcumin and vitamin D_3	Curcumin and Cholecalciferol in Treating Patients With Previously Untreated Stage 0-II Chronic Lymphocytic Leukemia or Small Lymphocytic Lymphoma	CLL	NCT02100423	Study is ongoing.
Calcium and vitamin D	Incidence of hematologic malignancy and cause-specific mortality in the Women's Health Initiative randomized controlled trial of calcium and vitamin D supplementation	All HM	PMID 28654155/ NCT00000611 (Women's Health Initiative)	Intervention lowered women's risk of HM incidence but not HM-specific mortality. Intervention was most protective for lymphoid malignancies.
Doxercalciferol	Phase II study of doxercalciferol for the treatment of myelodysplastic syndrome	MDS/CMML	NCT00052832	No hematological improvement was noted.

Fish oil	Oral fish oil positively influences nutritional-inflammatory risk in patients with haematological malignancies during chemotherapy with an impact on long-term survival: a randomised clinical trial	Unspecified leukemia and lymphomas	RBR-7q6cqg/28374923	Supplementation increased patient tolerance to chemotherapy resulting in a significant increase in overall survival.
Fish oil	Fish oil supplementation is beneficial on caloric intake, appetite and mid upper arm muscle circumference in children with leukaemia	Pediatric leukemia	PMID: 23017308	Treatment resulted in significant increase in mid-upper arm muscle circumference, and upward trend in appetite and caloric intake.
Milled seed mix	Dietary Milled Seed Mix in Patients With Non-Hodgkin Lymphoma	NHL	NCT03260231	Study is ongoing.
Maitake extract	Maitake mushroom extract in myelodysplastic syndromes (MDS): a phase II study	MDS	NCT01099917	The extract improved patient immune cell function.
Chia seed	Salvia Hispanica Seed in Reducing Risk of Disease Recurrence in Patients With Non-Hodgkin Lymphoma	NHL	NCT02652715	Study is ongoing.
Polyphenon E (EGCG)	Green Tea Extract in Treating Patients With Stage 0, Stage I, or Stage II Chronic Lymphocytic Leukemia	CLL	NCT00262743	Polyphenon E reversed and or slowed CLL progression.
SRT501 (Resveratrol) + Bortezomib	A Clinical Study to Assess the Safety and Activity of SRT501 Alone or in Combination With Bortezomib in Patients With Multiple Myeloma	MM	NCT00920556	SRT501 formulation of resveratrol was not well tolerated.

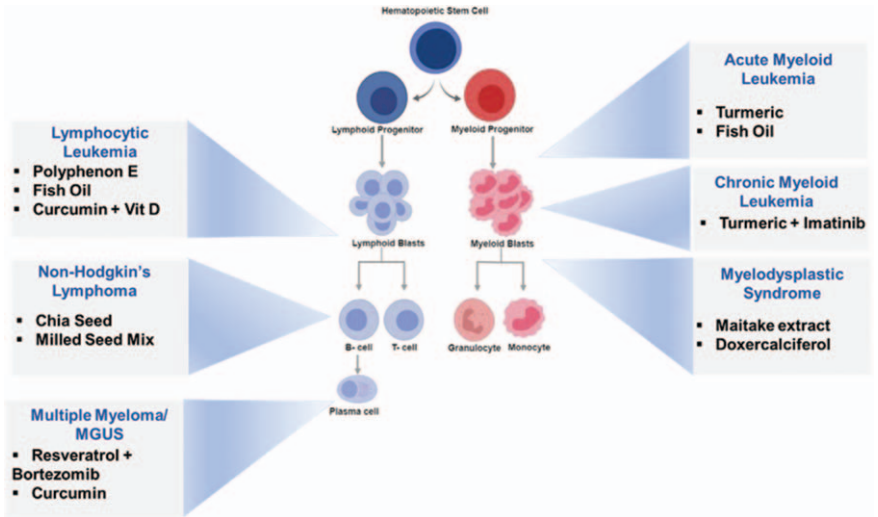

Figure 8.1 A simplified diagram describing the cell origin of various hematological malignancies and the nutraceuticals that have been investigated for their treatment.

non-Hodgkin's lymphomas (NHL) due to the absence of the Reed-Sternberg cells,[12] which are large (30–50 μm) multinucleated cells. In the US, NHL accounts for nearly 4–5% of new cancer diagnoses.[12] NHLs are further classified based on the cell of origin (either B, T, or NK cells) and the site where the malignant cells reside.[12,13] Similar to other lymphoid cancers, symptoms include lymphadenopathy, cytopenia, chronic fever, and weight loss.[12] Depending on the cell of origin and aggressiveness of the NHL subtype, a standard regimen consisting of **r**ituximab, **c**yclophosphamide, **h**ydroxydaunorubicin, **o**ncovin, and **p**rednisone (R-CHOP) is typically employed to manage disease progression.[13] Despite initial remission, drawbacks of R-CHOP include low cure rates and neutropenia, which leave patients vulnerable to infection.[13]

8.2.1.3 MGUS/Multiple Myeloma

Monoclonal gammopathy of undetermined significance (MGUS) is a common pre-malignant plasma cell disorder that can progress to multiple myeloma (MM), a more aggressive condition that causes severe organ damage.[14] MGUS has a prevalence rate of 4.2% in patients above 50 years of age.[13] The diagnosis and progression of MGUS is characterized based on several biomarkers that include $<30\ g\,L^{-1}$ (in serum) of paraprotein antibody fragments, decreased plasma cell populations in the bone marrow, and no organ damage.[15] MGUS is usually asymptomatic and is monitored but left untreated until it progresses to multiple myeloma.[15] Approximately 1% of MGUS patients progress to multiple myeloma, as determined by diagnostic biomarkers such as $>30\ g\,L^{-1}$ (in serum) of paraprotein, increased

populations of malignant and clonal plasma cells in the bone marrow, viscous blood and organ damage.[14,16] Organ damage includes osteolytic lesions, renal impairment, anemia, and increased susceptibility to infections.[16] Bortezomib, a chemotherapeutic that slows protein breakdown, and stem cell transplants may induce temporary remission but are unable to cure patients.[17] Long term regimens of bortezomib are not well tolerated as they cause myelosuppression and peripheral neuropathy.[17]

8.2.2 Myeloid Malignancies

Hematopoietic neoplasms of myeloid origin are broadly subdivided into myelodysplastic syndromes (MDS), chronic myeloid leukemia (CML) and acute myeloid leukemia (AML). As per the WHO classification of myeloid neoplasias and acute leukemias, ''myeloid'' encompasses all cells of the granulocyte, monocyte/macrophage, erythroid, megakaryocyte and mast lineages.[18] Among this group, the most common myeloid neoplasms are CML, MDS and AML.

8.2.2.1 CML

CML arises due to a clonal rearrangement that creates a fusion gene known as BCR-ABL1.[19] BCR-ABL1 leads to constitutive activation of protein tyrosine kinases which cause aberrant activation of various signalling transduction pathways.[19] Identification of this fusion gene has led to the development of specific tyrosine kinase inhibitors (*e.g.*, imatinib) that has vastly improved disease outcome for these patients leading to almost normal lifespans.[19] Despite this, resistance to kinase inhibitors can develop, leading to more unfavorable outcomes.[20]

8.2.2.2 MDS

MDS are classified as a dysplasia of at least one of the myeloid cell lineages paired with certain chromosomal abnormalities.[21] For a comprehensive review, see Corey *et al.*[22] In certain circumstances, MDS can progress to AML, the most common acute leukemia in adults.

8.2.2.3 AML

AML is a heterogeneous disease that results from the accumulation of various chromosomal translocations and genetic mutations.[23] Current AML induction therapy consists of the $7 + 3$ regimen, which is a 7-day continuous infusion of cytarabine followed by a 3-day infusion of an anthracycline.[24] In cases of relapsed or refractory AML, a hematopoietic stem cell transplant (HSCT) may be required to prolong patient survival. Although effective, it is a high-risk procedure and can often lead to the development of graft-*versus*-host disease that can lead to many comorbidities.

8.3 Nutraceuticals for Hematological Malignancies

8.3.1 Vitamin D

Vitamin D is a fat-soluble vitamin that has gained interest as an anti-neoplastic agent. It is obtained primarily from sun exposure and, to a lesser extent, the diet where it is metabolized in the liver to 1,25-dihydroxy-vitamin D [$1,25(OH)_2D$], a metabolite that binds to the nuclear vitamin D receptor (VDR).[25] Multiple leukemia and lymphoma cell lines express the VDR[25,26] and its activation can promote differentiation and apoptosis while slowing cancer cell cycling and proliferation.[26,27] In clinical studies, vitamin D supplementation has been shown to reduce prevalence and progression of hematological diseases. In a secondary analysis of the Women's Health Initiative, a randomized control trial ($N = 34\,763$) evaluating vitamin D and calcium supplementation, there was a decreased risk of developing a HM with supplement use with the greatest reduction in the lymphoid subtype.[28] Vitamin D deficiency, characterized by low plasma levels of $1,25(OH)_2D$, was associated with lower event-free survival for patients with CLL and lymphoid malignancies.[25,26] Likewise, in AML patients, low serum levels of 2(OH) vitamin D3 was associated with poorer patient outcomes.[29] Despite clinical studies that have demonstrated anti-proliferative effects of vitamin D, its development as an anti-cancer agent has been hindered by suboptimal dosing and occurrences of hypercalcemia in patients.[30] To circumvent this, some research groups have developed vitamin D analogues, while others have recommended intermittent dosing schedules, both of which are intended to prevent hypercalcemia while remaining effective.[31]

Doxercalciferol, a vitamin D analogue that maintains affinity for the VDR without causing hypercalcemia, was studied for its potential to treat MDS and chronic myelomonocytic leukemia (CMML). In a phase II study, 15 MDS/CMML patients were given 12.5 μg of doxercalciferol for 12 weeks and blood counts and toxicity were monitored.[32] After 12 weeks, doxercalciferol did not improve blood counts in MDS patients and even worsened counts for those MDS patients classified as having CMML. In the aforementioned Women's Health Initiative study, women who developed a HM despite supplementation had no difference in HM-related mortality compared to those who did not take vitamin D and calcium supplements. These studies suggest that the effect of vitamin D in hematopoietic neoplasms is still inconclusive and in need of further mechanistic and clinical studies.

8.3.2 Curcumin

Curcumin is the most notable bioactive compound derived from the rhizomes of tumeric (*Curcumin longa*). Turmeric and curcumin have a wide range of pharmacological properties that have been studied in a variety of diseases. *In vitro*, curcumin induces apoptosis in cell lines through various pathways including downregulating proliferation and pro-survival pathways,

such as hedgehog,[33] extracellular signal-regulated kinase (ERK),[34] wingless (Wnt),[33] Janus kinase/signal transducer and activator of transcription (JAK/STAT),[33] Notch,[33] and nuclear factor of kappa light polypeptide (NF-κB),[33] and by upregulating pro-apoptotic proteins, such as the Fas receptor (FasR),[35] tumor necrosis factor receptor (TNFR),[35] X-linked inhibitor of apoptosis (XIAP),[35] and certain members of the B cell lymphoma 2 (Bcl-2) family.[35]

Curcumin may also synergize with chemotherapy agents through its ability to inhibit telomerase activity or multi-drug resistance transporters.[33] The multidrug resistant transporter 1 (MDR1) codes for the P-glycoprotein, an ATP-dependent drug efflux pump.[36] Certain subtypes of CML possess polymorphisms of MDR1, leading to increased P-glycoprotein activity, drug efflux and imatinib resistance. Curcumin has been shown to sensitize BCR-ABL-positive cells to imatinib-induced apoptosis through suppression of MDR1 expression and P-glycoprotein activity.[37] A clinical study was conducted to test the efficacy of imatinib in combination with turmeric powder in improving disease measures of CML patients. In this study, patients were divided into two groups and given either imatinib alone (400 mg, twice daily) or with turmeric powder (5 g, three times a day) for 6 weeks. Hematological response (cell counts and bone marrow morphology) and nitric oxide (NO) levels, which is linked to tumorigenesis,[38,39] were used as markers of disease progression. NO levels were significantly reduced in both groups, but the effect was more pronounced in the combination group.[37] In addition, patients receiving the combination therapy reported a greater hematological response and fewer side-effects than imatinib alone; however, this result was not statistically significant.

A daily oral intervention of curcumin was recently explored as a strategy to slow progression of MGUS to multiple myeloma,[40] of which a hallmark sign is the formation of osteolytic lesions.[41] In cell culture, curcumin reduced multiple myeloma cell proliferation and induced apoptosis[40] *via* the suppression of the receptor activator of nuclear factor kappa-B ligand (RANKL), which upregulates osteoclastogenesis and slows bone degradation.[34,40] For this study, MGUS patients with $<30\ g\,L^{-1}$ of serum paraprotein consumed a daily tablet containing 4 g of curcumin or a placebo for 6 months. Disease progression was monitored by paraprotein concentrations and levels of urinary N-telopeptide of type I collagen (uNTx), a marker of bone resorption.[40] At study termination, those on curcumin had decreased levels of paraprotein (by 12–30%) and uNTx (by ~25%).[40] While differences were noted in overall levels, the trial ultimately found no statistically significant differences between treatment and placebo groups.[40]

Curcumin's bioactivity is limited by its hydrophobicity and is a significant issue in product formulation. For example, the mild-to-moderate activity summarized above is observed by consuming significant amounts (*i.e.*, 4–15 g) of powder per day! As such, several labs have developed novel formulations to circumvent this issue and improve curcumin's efficacy.[42,43] In cell culture, a formulation of curcumin encapsulated by polylactic-*co*-glycolic acid (PLGA)

allowed for more rapid and efficient leukemia cell absorption.[42,43] Following intravenous administration, the maximum serum concentration of the PLGA-curcumin was almost twice that of non-formulated curcumin.[43] With improved solubility and release, this conjugated formulation was more effective at inhibiting drug efflux pumps, slowing development of chemoresistance, downregulating NF-kB, and inducing apoptosis of leukemic K562 cells.[42,43] As such, future trials should be focused on these advanced formulations.

8.3.3 Green Tea Bioactives

Green tea and its active constituents, which are predominantly catechins and polyphenols, can induce apoptosis and synergize with chemotherapeutics in cancer cell lines and animal models.[44,45] Secondary analysis of a Japanese cohort study ($N = 41\,761$ participants) found that green tea consumption significantly reduced the risk of developing lymphoid and myeloid neoplasms.[46] Green tea did not however reduce the risk of solid tumors, suggesting that neoplasms of hematological origin possess unique characteristics that are impacted by green tea bioactives.[46] A bioactive of particular interest is epigallocatechin gallate (EGCG), which modulates targets critical to cancer cell survival and migration such as mitogen activated protein kinases (MAPK),[47] activator-protein 1 (AP-1),[47] vascular endothelial growth factor (VEGF),[48] and Bcl-2 proteins.[48] EGCG also increases transcription of p53, a critical tumor suppressor controlling genes associated with cell cycle arrest and apoptosis.[49] Of these targets critical to CLL progression, VEGF and Bcl-2 proteins are consistently expressed at high levels and are associated with a poor prognosis.[50,51] In addition, MAPK and AP-1 in CLL are constitutively upregulated to support uncontrolled proliferation and resistance to chemotherapy-induced apoptosis.[52]

A Phase 1/2 trial was conducted to determine the efficacy of Polyphenon E, a standardized extract of green tea catechins, in patients with asymptomatic CLL who had not progressed enough to start chemotherapy.[53] Patients consumed 4 g of Polyphenon E or a placebo daily for up to 6 months[53] and absolute lymphocyte count (ALC) and incidence of lymphadenopathy, two markers of CLL progression, were monitored.[53] Polyphenon E was well-tolerated and resulted in a sustained $> 20\%$ decrease in ALC in 30% of patients and a $> 50\%$ reduction in lymphadenopathy in over half of those who completed treatment.[53] These decreases in CLL clinical markers suggest Polyphenon E slows CLL progression in patients who have not initiated chemotherapy.[53]

8.3.4 Omega-3s

Omega-3 polyunsaturated fatty acids (PUFAs) are involved in many physiological pathways such as cell signalling and plasma membrane integrity.[54] Their consumption has been linked to disease prevention and, in HMs,

they have been shown to induce apoptosis through various mechanisms that include modification of intracellular calcium levels and membrane permeability.[55]

Fish oils are a rich source of the omega-3 PUFAs docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). In a randomized controlled trial of 22 adults with newly diagnosed leukemia or lymphoma, patients were given fish oil capsules (2 g per day for 9 weeks) concomitantly with chemotherapy.[56] Patients given fish oil were able to endure more rounds of chemotherapy and had significantly increased overall survival.[56] Fish oil has also been studied for the prevention of chemotherapy-induced malnutrition and weight loss. Patients (4–12 years old; $N = 51$) with leukemia were given a 1200 mg fish oil capsule daily for 60 days and measures of caloric intake, appetite, and mid-upper arm muscle circumference (MUAMC) were measured.[57] After 60 days, fish oil supplementation significantly increased MUAMC. In addition, patients receiving fish oil showed a trend toward an increase in overall body weight, caloric intake, and appetite; however, these were not statistically significant.[57] Together, these results suggest that fish oil supplementation may help to improve the nutrition status of leukemic patients undergoing chemotherapy.

Compared to healthy controls, patients with progressive NHL showed abnormal lipid profiles including decreased levels of total phospholipids (PLs), increased levels of saturated fatty acids (SFA), and decreased levels of omega-3 unsaturated fatty acids.[58] These negative prognostic indicators were associated with accelerated disease progression and decreased long-term patient survival.[58] Studies in cell lines and rodent models have shown that the number and location of un-saturations on a PL may also modulate NHL response to chemotherapy.[58] Post-chemotherapy profiling showed that patients who experienced disease progression continued to have low PL levels while patients achieving remission had increased PL levels.[59,60] Given this emerging importance of PL as a prognostic factor in NHL, two ongoing pilot studies are determining how PUFA-rich seed mixes can alter PL profiles and slow NHL progression.[61,62]

In healthy cohorts, increased consumption of dietary omega-3 fatty acids was associated with a transient improvement in gut microbiome diversity.[63] Several studies have noted the presence of pathogenic gut bacteria and a decrease in gut microbiome diversity in HL and NHL patients, compared to healthy controls.[64,65] In rodent models, pathogenic bacteria such as *Helicobacter pylori* and *Campylobacter jejuni* were shown to directly initiate lymphomagenesis through the constant stimulation of immune cells; this chronic and constant antigen presentation leads to continuous B cell expansion and an unwanted release of pro-inflammatory cytokines such as interleukin-6 and tumor necrosis factor α.[66] In an ongoing study where chia seeds are consumed daily for 12 weeks, NHL patients currently in remission are monitored for disease recurrence as well as changes in serum levels of alpha-linoleic acids and fecal microbiome composition.[62]

These studies suggest that omega-3 PUFAs may improve nutritional status and quality of life of HM patients creating greater tolerance for chemotherapy. Despite positive results, additional rigorous randomized controlled trials must be conducted to support these preliminary findings and to determine whether omega-3 PUFAs synergize or antagonize with current chemotherapeutics.

8.3.5 β-Glucans

β-Glucans are polysaccharides derived from bacteria, fungi, and cereals such as oats and barley. Anti-neoplastic effects of β-glucans have been studied in MDS and multiple myeloma.[67] In MDS, pre-clinical studies demonstrated that β-glucans derived from the maitake mushroom promoted differentiation and granulocyte colony-stimulating factor production.[68] These results were corroborated in a non-randomized, open-label phase II clinical trial. Here, 18 low-risk MDS patients given 3 $mg\,kg^{-1}$ maitake extract twice daily for 12 weeks had improved peripheral blood monocyte and neutrophil function, relative to the start of the trial, and the extract was well tolerated.[69]

8.3.6 Resveratrol

Resveratrol is a stilbene (*i.e.*, phenol) derived from various nuts and berries and is most notably found in red grapes. *In vitro* studies reported that resveratrol reduces proliferation and synergizes with bortezomib to induce apoptosis of myeloma cells through mechanisms related to increasing expression of sirtuin-1,[70] decreasing activity of NF-κB and inhibiting signal transducer and activator of transcription 3 (STAT3) pathways.[71,72] In 2012, a Phase 2 trial investigated the efficacy of resveratrol with or without bortezomib in relapsed multiple myeloma patients. Patients were placed on a monotherapy of SRT501 or in combination with bortezomib. SRT501 is a micronized formulation designed to improve resveratrol's inherently poor bioavailability and water solubility.[73] Patients were to be upgraded from monotherapy to the combination regimen if the disease progressed.[73] All patients in the monotherapy group were eventually upgraded to the combination therapy or left the study due to severe adverse events.[73] In fact, 45% of the monotherapy group withdrew from the study after reporting adverse events such as nausea, diarrhea, vomiting, fatigue, anemia, renal failure, and infection.[73] By study end, 8% of the group receiving combination therapy experienced either a partial or minor response based on multiple myeloma prognostic markers, bone marrow plasma cells counts, and skeletal X-ray examinations. The high incidence of adverse events and minimal improvement in disease condition suggests that the SRT501 formulation was not well-tolerated or effective.[73] While preclinical studies continue to confirm resveratrol's strong anti-cancer potential, alternative formulations that focus on efficacy and minimizing dose-limiting toxicities should continue to be the focus of future research.

8.3.7 Ascorbate

Ascorbate (*i.e.*, vitamin C), obtained entirely from dietary sources, directly modulates hematopoietic function and leukemic progression.[74] During healthy hematopoiesis, ascorbate regulates HSC function, frequency, and differentiation. It is a co-factor of tet methylcytosine dioxygenase 2 (TET2), an enzyme responsible for oxidizing 5-methylcytosine to 5-hydroxymethylcytosine (5-hmc) to modulate DNA methylation.[75] In murine models, ascorbate depletion decreased TET2 activity, decreased 5-hmc levels and increased HSC frequency while ascorbate supplementation restored TET2 activity and promoted erythrocyte differentiation.[74] Ascorbate depletion also co-operates with mutations such as Flt3-ITD to accelerate leukemic transformation while ascorbic supplementation suppresses this leukemic progression.[74] Further, metabolomic assessment determined that plasma ascorbate levels were significantly decreased in leukemia patients, compared to healthy controls.[74]

Use of ascorbate as a chemotherapeutic has been revisited after several pharmacokinetic studies demonstrated a >6-fold increase in plasma ascorbate following intravenous *versus* oral administration.[76] A Phase I clinical trial reported that a combination of 20 $mg\,m^{-2}$ per day decitabine, 0.2 $mg\,kg^{-1}$ per day arsenic trioxide, and 1000 mg per day ascorbate was well-tolerated in 13 patients with AML or MDS. After four cycles of the Phase I study, one patient entered remission and five others showed stable disease.[77] A second Phase I clinical trial in patients with stage 3 relapsed multiple myeloma reported that 0.25 $mg\,kg^{-1}$ per day arsenic trioxide with 1000 mg per day ascorbate was well-tolerated across six patients; two relapsed patients previously treated with thalidomide showed a 50% reduction in marrow plasma cells and four other patients showed stable disease.[78] Together, these results demonstrate the promise of ascorbate in HM and provide the foundation to support larger trials.

8.4 Conclusion

All chemotherapeutics used in HM have unintended, harsh side-effects that adversely affect a patient's quality of life.[79] Increasing numbers of cancer patients and survivors are turning to nutraceuticals in an attempt to either speed recovery from chemotherapy side effects or prevent relapse.[80] Nutraceuticals are believed to be safe since they are derived from food[79] and patients routinely consume these compounds without informing primary caregivers who are also administering conventional chemotherapeutics.[79] However, there have been several well-documented cases of antagonism between chemotherapeutics and nutraceuticals in HMs. For example, EGCG and vitamin C both hinder the action of bortezomib, a standard chemotherapeutic prescribed for lymphomas and multiple myeloma.[81,82] Nutraceuticals may have clinical efficacy either alone or in combination with current chemotherapeutics for patients with a HM; however, additional clinical studies are needed to better define their role in cancer chemotherapy.

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CHAPTER 9

Nutraceuticals as Metabolic Modulators for the Treatment of Obesity and Associated Diseases

NAWAZ AHMED, SARAH WALKER AND PAUL A. SPAGNUOLO*

Department of Food Science, University of Guelph, 50 Stone Rd., Guelph, Ontario, Canada N1G2W1
*Email: paul.spagnuolo@uoguelph.ca

9.1 Introduction

Obesity is a global pandemic spreading at an alarming rate[1] and is strongly related to the soaring rates of other metabolic diseases such as Type 2 Diabetes (T2D) and heart disease. Insulin resistance is a clinical hallmark of T2D defined by the inability of cells in metabolically active tissues to respond to insulin, leading to glucose intolerance and lethal vascular complications. In diet-induced obesity (DIO), insulin resistance can result from excess amounts of circulating plasma free fatty acids (FFAs) accumulating in non-adipose tissues such as skeletal muscle, liver, and pancreas.[2]

The optimal metabolic health of a cell is governed by the ability of mitochondria to switch freely between oxidative substrates in response to physiological and nutritional cues. Mitochondrial dysfunction in key

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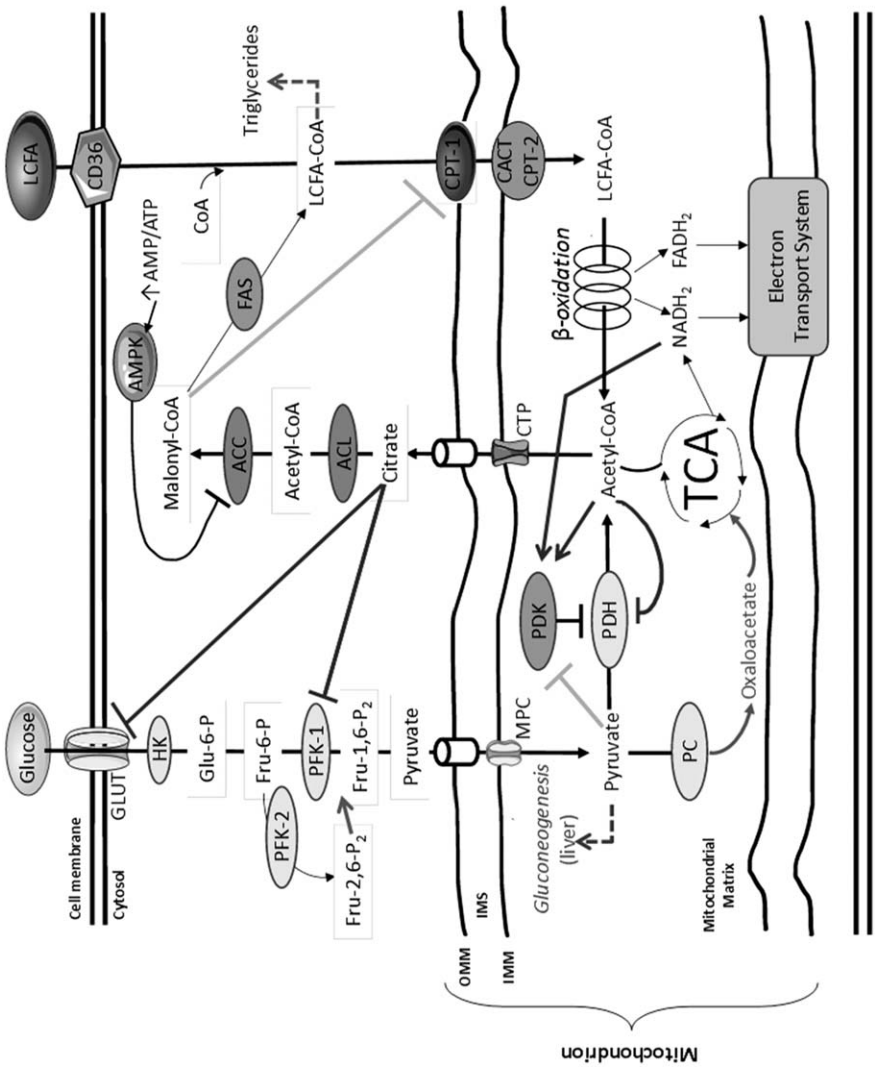
tissues is therefore believed to be at the centre of obesity and associated disorders, which are now denoted by a new clinical hallmark: metabolic inflexibility. Metabolic inflexibility is defined as the inability of an organism or a single cell to adapt fuel oxidation to fuel (*i.e.*, substrate) availability[3] that results in lower glucose oxidation during insulin-stimulated conditions. The importance of metabolic inflexibility is highlighted by numerous studies showing that oversupply of FFAs in metabolically active tissues overloads the mitochondria leading to incomplete fatty acid oxidation (FAO) and increased oxidative stress that worsens and/or perpetuates insulin resistance.[4–11] In light of this emerging evidence, strategies that target mitochondrial fuel selection by modulating lipid and glucose metabolism have been proposed as therapies to improve insulin resistance.[12–15] While lifestyle interventions (*e.g.*, diet, exercise and stress management) are undoubtedly the most economical treatment and prevention options, they have failed to make a lasting impact at the population level. This highlights the need for pharmacotherapy as an adjunct to lifestyle interventions; however, available drugs vary in efficacy, side-effect profiles, and are often inaccessible due to inadequate health-insurance coverage.[16] The search for alternative treatments for these diseases has led to the discovery of natural health products (NHPs), derived from plants or food, that are currently being used for the management of obesity and associated metabolic disorders.[17] This chapter will aim to examine a handful of NHPs that have the potential to act as metabolic modulators that restore impairments in mitochondrial lipid and glucose metabolism in obesity and associated disorders.

9.2 Metabolic Flexibility in Health

The metabolism of a cell or tissue must be able adapt to fluctuations in energy demand or substrate availability to survive. Metabolic flexibility is evident in how healthy tissues adapt to changing conditions such as going from a fasting-to-fed state, rest-to-exercise, and even to changes in circadian rhythm.[3,18] The concept of metabolic flexibility was first coined in 1963 when the Randle Cycle was proposed as an explanation for the relationship between FAO (or β-oxidation) and glucose oxidation.[19] Randle and colleagues discovered that while the insulin : glucagon ratio from the fasting-to-fed states controls anabolic *versus* catabolic metabolism, there is a finer level of hormone-independent metabolic control.[19] This is the relationship between fatty acid and glucose oxidation where the oxidation of one substrate directly inhibits the oxidation of the other.[19] For example, going from a fasting-to-fed state involves a shift in substrate utilization from fatty acids to glucose in skeletal muscle tissue.[3] The increased oxidation of glucose is, of course, driven by insulin but the subsequent down regulation of FAO is a result of the glucose–fatty acid cycle.[3] In the fasted state, fat stores are broken down to provide FFAs as metabolic fuel substrates.[19] This inhibits


Glucose
GLUT
Cell membrane
Cytosol
HK
Glu-6-P
Fru-6-P
PFK-1
PFK-2
Fru-2,6-P2
Fru-1,6-P2
Pyruvate
LCFA
CD36
AMP/ATP
AMPK
Malonyl-CoA
ACC
Acetyl-CoA
ACL
Citrate
CoA
FAS
Triglycerides
LCFA-CoA
CPT-1
CACT
CPT-2
LCFA-CoA
β-oxidation
FADH2
NADH2
Electron Transport System
CTP
Acetyl-CoA
TCA
PDK
PDH
MPC
Pyruvate
PC
Oxaloacetate
Gluconeogenesis
(liver)
Mitochondrial Matrix
OMM
IMS
IMM
Mitochondrion

glucose oxidation and, importantly, preserves glucose for tissues that depend on it (*i.e.*, brain) until the next meal is consumed.[19] During exercise, skeletal muscle tissues need metabolic flexibility to adapt to increased energy demands where intensity and duration of exercise dictates the substrates that are utilized.[3]

On a systemic level, the biochemical transitions described above rely on endocrine signalling between multiple organs which coordinate control of available fuel. Figure 9.1 highlights cellular components of the glucose–fatty acid cycle where allosteric inhibition of key regulatory enzymes in opposing metabolic pathways enables a rapid switch in substrate oxidation. In the post-prandial period of a glucose rich meal, blood glucose and insulin levels spike causing glucose uptake, glycolysis, and subsequent pyruvate oxidation to acetyl Co-A in the mitochondria *via* the pyruvate dehydrogenase (PDH) complex (*i.e.*, the first key regulatory enzyme complex). Additionally, the increase in pyruvate from glycolysis inhibits pyruvate dehydrogenase kinase (PDK), which further activates the PDH complex, increasing glucose oxidation.[20] These glucose-dependent processes increase the concentration of the cytosolic substrate malonyl-CoA, which allosterically inhibits carnitine palmitoyltransferase-1 (CPT-1). CPT-1 is a key regulatory mitochondrial enzyme that controls entry of long-chain fatty acids into the mitochondria for FAO. Inhibition of CPT-1 *via* malonyl-CoA generally re-routes fatty acids towards triglyceride synthesis and storage. Conversely, during fasting, glucose-mediated inhibition of FAO is released by the action of the energy stress sensor adenosine monophosphate-activated protein kinase (AMPK), which inhibits acetyl-CoA carboxylase (ACC).[20] Inhibition of ACC lowers malonyl-CoA concentrations and results in increases in CPT-1 activity and the rate of mitochondrial β-oxidation. FAO increases mitochondrial acetyl Co-A and reduces nicotinamide adenine dinucleotide (NADH) levels, which collectively inhibit PDH activity *via* both allosteric inhibition and PDK activation.[20] Additionally, an increase in fat availability elevates cytosolic

Figure 9.1 The glucose–fatty acid cycle. Light grey lines show glucose-dependent processes suppressing FAO and dark grey lines show FAO processes inhibiting glucose oxidation. LCFA, long chain fatty acid; CD36, cluster of differentiation 36 (membrane fatty acid transporter protein); LCFA-CoA, long-chain fatty acyl-coenzyme A; CPT-1, carnitine palmitoyltransferase-1; CACT, carnitine acylcarnitine translocase; NADH; nicotinamide adenine dinucleotide reduced form; FADH; flavin adenine dinucleotide reduced form; CTP, citrate transport protein; ACL, ATP-citrate lyase; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; AMPK, adenosine monophosphate-activated protein kinase; GLUT, glucose transporter; HK, hexokinase; Glu-6-P; glucose 6-phosphate; Fru-6-P; fructose 6-phosphate; PFK, phosphofructokinase; OMM, outer mitochondrial membrane; IMS, intermembrane space; IMM, inner mitochondrial membrane, MPC, mitochondrial pyruvate carrier; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase complex; PDK, pyruvate dehydrogenase kinase; TCA, tricarboxylic acid cycle.

citrate levels that inhibit glucose transporters (GLUT) and the glycolytic enzyme phosphofructokinase-1 (PFK-1), which collectively impede glucose uptake and utilization.[20] As mentioned above, this allosteric inhibition of glucose oxidation *via* FAO processes helps to conserve glucose for anabolic pathways and brain metabolism. Importantly, when FAO inhibits glucose oxidation, a decrease in pyruvate oxidation enables its use as a substrate for pyruvate carboxylase (PC) in energetically demanding tissues, which produces oxaloacetate that feeds into the tricarboxylic acid (TCA) cycle and acts as an anaplerotic substrate. In the liver, however, lower pyruvate oxidation (due to elevated FAO) leads to the use of pyruvate as a precursor for gluconeogenesis (production of glucose from non-carbohydrate sources) to avoid hypoglycemia;[20] this pathway becomes extremely problematic in obesity and T2D as will be explained in the next section.

9.3 Obesity, Lipotoxicity and Metabolic Inflexibility

The imbalance between energy intake and expenditure in obese individuals leads to expansion of adipose tissue deposits. Adipose tissue expansion is a physiological and protective process[21] that is heavily impaired in obesity. The inability of adipose tissue to store FFAs from systemic circulation causes overflow and accumulation into non-adipose tissues (termed ectopic fat) such as muscle, liver, and pancreas.[22,23] Ectopic fat can lead to lipotoxicity. In lipotoxicity, tissue mitochondria are overloaded and FAO is chronically ramped up without co-ordinated increases in TCA cycle or electron transport chain fluxes. This ultimately lead to incomplete FAO, which is characterized by a build-up of intra-mitochondrial toxic lipid intermediates and free radicals that cause insulin resistance and impairment in organ function.[7] Figure 9.2 highlights key metabolic impairments in an obese and insulin-resistant individual.

Clinical observations in obese and insulin-resistant individuals show that insulin is unable to efficiently supress FAO or sufficiently stimulate glucose oxidation (Figure 9.2). Thus, there is an impairment in switching between fat and glucose oxidation in fasting and insulin-stimulated conditions, respectively.[24] The molecular mechanisms of obesity and T2D related metabolic inflexibility in different tissue types is a topic of great debate and continued research. It is unclear which is the first organ to become metabolically inflexible, or whether metabolic inflexibility precedes or proceeds insulin resistance. There is, however, strong consensus on the molecular mechanisms of lipotoxicity and metabolic inflexibility in skeletal muscle.[7] This mechanism is highlighted in Figure 9.3 and shows how insulin resistance in skeletal muscle is characterized by excessive β-oxidation in the post-prandial state. Excessive β-oxidation gives rise to incomplete FAO, which produces lipotoxic lipid intermediates that impair glucose utilization and insulin signalling, deplete organic intermediates of the tricarboxylic acid (TCA) cycle, generate excessive reactive oxygen species (ROS) and alter

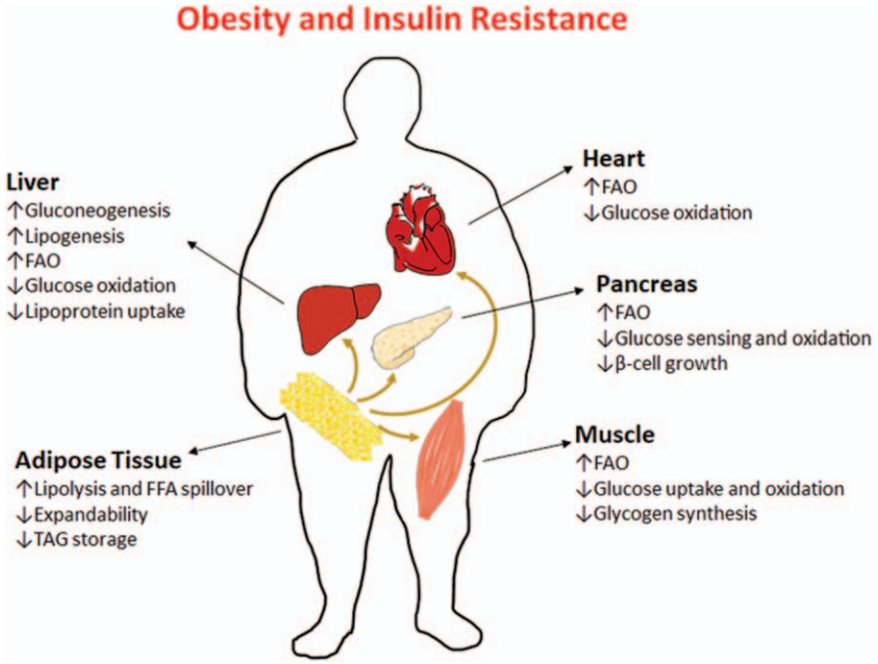
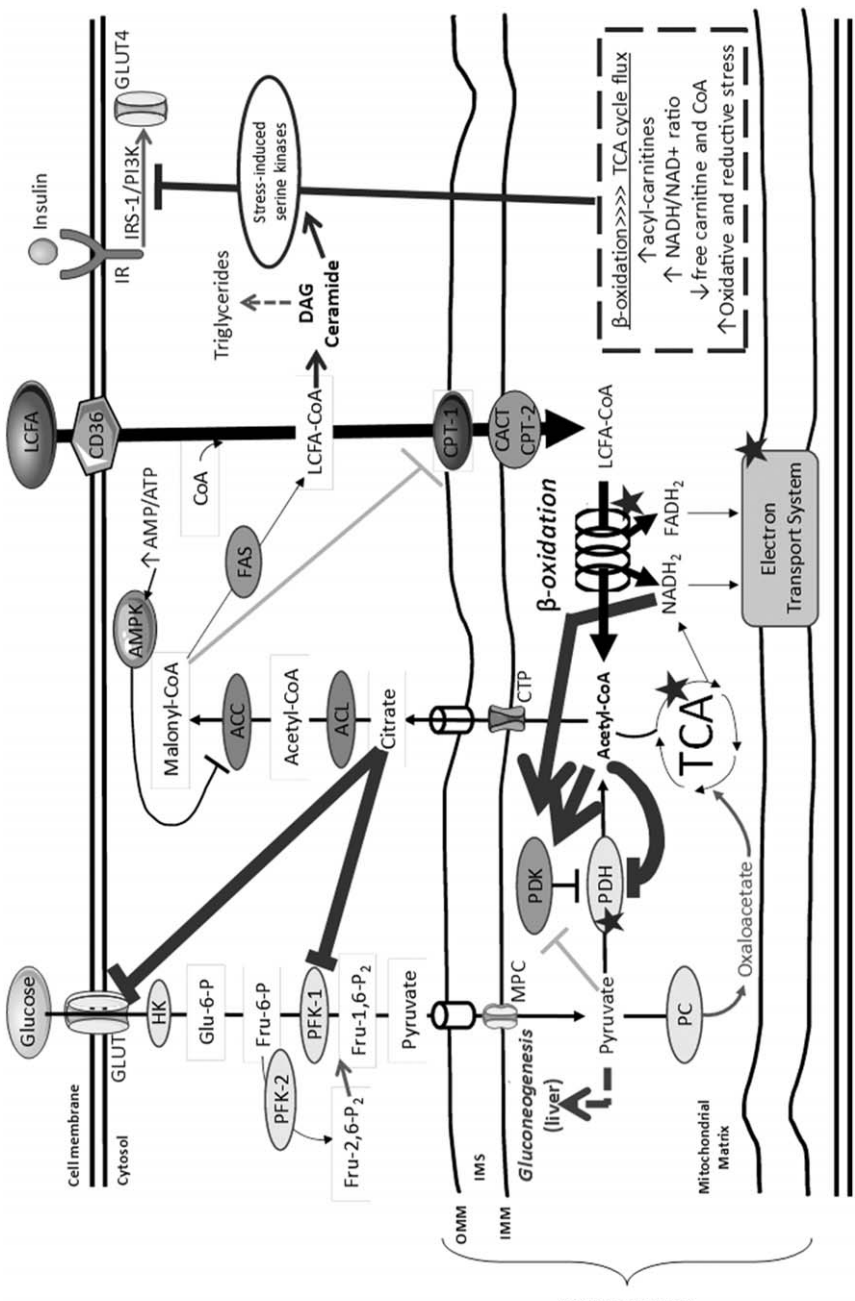


Figure 9.2 Lipotoxicity in an obese and insulin-resistant adult. Caloric excess and a sedentary lifestyle reduces expandability of adipose tissue, which causes spillover of FFA to non-adipose tissue (brown arrows) leading to insulin resistance in liver, muscle, heart and pancreas. Insulin resistance in non-adipose tissue leads to key metabolic impediments (as highlighted in the figure) that cause whole body metabolic inflexibility and enables the onset of further complications like T2D and cardiovascular diseases.

mitochondrial protein acetylation states. This cascade of events chronically impedes mitochondrial performance and oxidative capacity.[7]

Several elements of the mechanism of lipotoxicity and metabolic inflexibility in skeletal muscle have also been observed in other non-adipose tissues. In the pancreas, the site of insulin secretion and synthesis, lipotoxicity or increased FAO contributes to the pathogenesis of T2D by causing dysfunction and apoptosis of insulin-producing β-cells.[25] In pre-diabetes, where β-cell metabolic dysfunction instead of apoptosis is more prominent, FFA-induced lipotoxicity disrupts the glucose–fatty acid cycle[26,27] and impairs glucose-stimulated insulin secretion (GSIS), resulting in hyperinsulinemia.[26,28–30] During ischemic heart disease, blood supply is restricted to cardiac tissue, resulting in a lack of oxygen for oxidative phosphorylation and an increased reliance on FAO.[3,31] In this scenario, insufficient energy production is not due to lack of available substrate but rather to metabolic inflexibility resulting from a lack of oxygen—a key distinction from the mechanism in skeletal muscle. Here, metabolic flexibility is severely impaired in the ischemic and post-ischemic heart (as well as in


Insulin
IR
IRS-1/PI3K
GLUT4
Stress-induced serine kinases
Triglycerides
DAG
Ceramide
LCFA
CD36
LCFA-CoA
CPT-1
CACT
CPT-2
LCFA-CoA
β-oxidation
β-oxidation>>>> TCA cycle flux
↑acyl-carnitines
↑ NADH/NAD+ ratio
↓free carnitine and CoA
↑Oxidative and reductive stress
AMPK
↑AMP/ATP
CoA
FAS
Malonyl-CoA
ACC
Acetyl-CoA
ACL
Citrate
CTP
Acetyl-CoA
NADH2
FADH2
Electron Transport System
TCA
Glucose
GLUT
HK
Glu-6-P
Fru-6-P
PFK-2
PFK-1
Fru-2,6-P2
Fru-1,6-P2
Pyruvate
PDK
PDH
MPC
Pyruvate
PC
Oxaloacetate
Gluconeogenesis (liver)
Cell membrane
Cytosol
Mitochondrial Matrix
OMM
IMS
IMM
Mitochondrion

angina and heart failure), as cardiac muscle cells are exposed to excess circulating FFAs in amounts far greater than their oxidative capacity. As such, cardiac muscle cells become locked in a fatty acid dependent state where 60–90% of energy is derived from FAO[32]—a far less efficient metabolic pathway yielding less ATP per molecule of oxygen compared to glucose oxidation. Excess FAO during and post-ischemia perpetuates ischemic damage by (1) reducing glucose oxidation, (2) causing mitochondrial dysfunction and oxidative stress, and (3) exacerbating ischemia-induced ionic imbalance.[33] In the liver, insulin resistance perpetuates lipid accumulation, which is clinically observed as a hallmark of non-alcoholic fatty liver disease (NAFLD). In NAFLD, hepatic uptake of lipids and *de novo* lipogenesis are increased causing a compensatory enhancement of FAO.[34] Increased FAO is insufficient in normalizing lipid levels and instead promotes oxidative stress, gluconeogenesis and metabolic inflexibility.[35]

9.4 Metabolic Modulators and Modes of Modulation

Obesity and associated metabolic disorders share a common underlying pathological event characterized by alterations in fatty acid metabolism that impairs glucose metabolism. Habitual exercise has been shown to combat this mitochondrial lipid overload by increasing TCA cycle flux and mitochondrial biogenesis, thereby restoring mitochondrial function and enhancing muscle insulin sensitivity.[36] However, if exercise cannot be easily implemented, a potential treatment strategy is to target mitochondrial dysfunction by directly or indirectly modulating fatty acid and/or glucose metabolism. Pharmacological agents as metabolic modulators have been researched primarily in the field of heart disease and a number of these agents are now in clinical trials or clinically available. Based on current preclinical and clinical evidence, Figure 9.4 highlights the main modes by which metabolic modulation can be achieved in a variety of

Figure 9.3 Molecular mechanism of lipotoxicity and metabolic inflexibility in skeletal muscle. Caloric excess and inactivity result in a surplus of fatty acids in mitochondria that promotes β-oxidation without a co-ordinated increase in TCA cycle flux. Chronic lipid overload leads to an increase in redox potential and depletion of free CoA and carnitine. Together this exerts negative pressure on the TCA cycle and electron transport chain while also inhibiting glucose disposal through PDH. The increased oxidative and reductive stress disrupts mitochondrial performance and alters the acetylation state of mitochondrial proteins (stars). As metabolic by-products of incomplete FAO accumulate (*e.g.*, acylcarnitines, NADH, ROS), LCFA-CoAs are rerouted towards triglycerides synthesis; however, the mitochondrial overload causes cytosolic build-up of DAGs and ceramides. Collectively, mitochondrial and lipid-derived stresses activate serine kinases that block insulin receptor (IR) signaling, GLUT4 translocation (through the IRS-1/PI3K pathway) and glucose metabolism. DAG, diacylglycerol; IR, insulin receptor; IRS-1, insulin receptor substrate-1; PI3K, phosphoinositide 3-kinase. Data from Koves *et al.*[7]

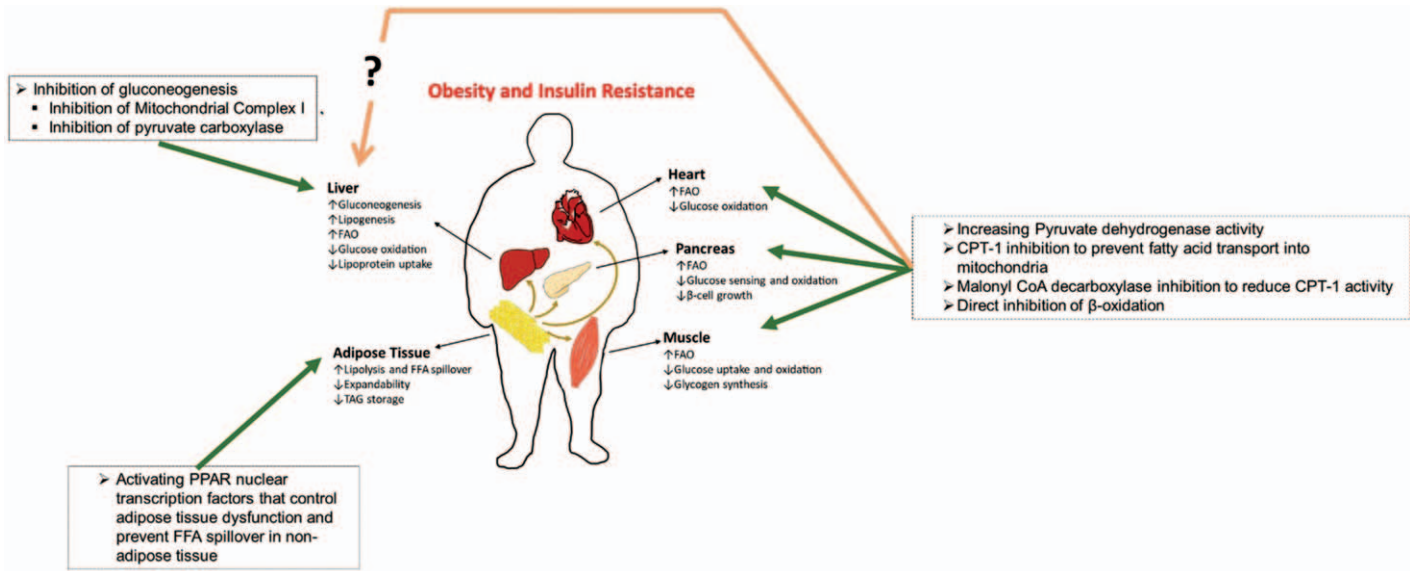


Figure 9.4 Modes of metabolic modulation that spares or improves glucose oxidation in metabolically active tissues affected by obesity-associated lipotoxicity.

tissues that are affected by lipotoxicity in obesity and associated metabolic diseases.

One of the biggest successes for metabolic modulators is trimetazidine, which is currently approved as a treatment for angina pectoris. It acts by inhibiting the final enzyme of β-oxidation, thereby decreasing FAO and leading to a subsequent increase in glucose oxidation in myocardial tissue.[37] Trimetazidine upregulates PDH activity in cardiomyocytes and helps re-couple glucose oxidation to glycolysis.[37] It is also involved in restoring function in mitochondria damaged during ischemia and directly inhibits cardiac fibrosis.[37] There are few larger scale clinical trials testing trimetazidine; however, smaller trials and meta-analyses demonstrate its safety and, since it does not decrease resting blood pressure, it can be used with standard treatments like beta blockers.[38] Ranolazine is another metabolic modulator approved for use in angina pectoris that is showing promising results in patients with heart failure.[39] Although initially proposed as a partial FAO inhibitor (*i.e.*, causes modest (~40–50%) FAO inhibition[40]), it imparts activity mainly by restoring ionic homeostasis during heart failure.[41] Etomoxir is a direct inhibitor of CPT-1 and is typically used to study FAO experimentally, as it exhibited severe dose-limiting liver toxicity in clinical trials.[42] Perhexiline is also a CPT-1 inhibitor with a narrow therapeutic window (*i.e.*, potential hepato- and neuro-toxicity) that has shown clinical promise for heart failure.[38,43,44] There are no additional classes of FAO inhibitors currently being evaluated in clinical trials for ischemic heart disease; however, compounds that inhibit malonyl CoA decarboxylase, an enzyme that catalyzes the conversion of malonyl CoA to acetyl CoA thereby reducing CPT-1 activity, are showing preclinical promise in mouse models of heart failure.[45]

Unlike for heart disease, pharmacological modulators of metabolism have yielded limited clinical success when evaluated as treatments for obesity and T2D. While the beneficial effects of trimetazidine as a partial FAO inhibitor in heart muscle are well established, its efficacy in the treatment of obesity and diabetes is hampered due to its limited activity in skeletal muscle.[46] Ranolazine has recently been approved for use in combination therapies for the treatment and management of T2D; however; its beneficial activity is not due to FAO inhibition in skeletal muscle.[47] Recently, ranolazine was demonstrated to increase liver PDH activity, which reduced fat accumulation, body weight gain and glucose intolerance in a mouse model of obesity.[48] Future clinical studies are likely to elucidate its mechanism of action in obese and diabetic humans. The unfavorable safety profile of etomoxir has limited the clinical development of CPT-1 inhibitors for the treatment of diabetes and obesity;[49] nonetheless, recent efforts in pilot clinical studies have reported favorable effects of etomoxir with dose adjustments.[50] A CPT-1 inhibitor called Teglicar, a reversible and selective inhibitor of liver-specific CPT-1, demonstrated impressive pre-clinical results where it significantly lowered fasting blood glucose, insulin resistance, and postprandial gluconeogenesis in diabetic rats;[51] Phase I

clinical trial results are not yet published. In the 1990s, thiazolidines (TZDs) were introduced as a new class of drug for the treatment of T2D. TZDs (*e.g.*, rosiglitazone) are agonists for the transcription factor peroxisome proliferator-activated receptor gamma (PPAR-γ). The activation of PPAR-γ in adipose tissue leads to adipogenesis that reduces ectopic fat accumulation in other tissues and can restore metabolic flexibility. TZDs currently on the market have known adverse side-effects like cardiac toxicity, hepatotoxicity, and gastric discomfort, which hinders patient compliance, impacting overall effectiveness.[52] Lastly, malonyl CoA decarboxylase inhibitors[53] and a novel class of peptide-based CPT-1 inhibitors[54] have shown preclinical promise in mouse models of obesity but are far from clinical translation.

9.5 Natural Health Products (NHPs) and Nutraceuticals as Metabolic Modulators

Here we will review a handful of NHPs and nutraceuticals that can act as classic metabolic modulators, *i.e.*, agents that directly act on the mitochondria to modulate glucose and lipid metabolism thereby improving metabolic flexibility. We refer our readers to a recent review by Serrano *et al.*[55] that summarizes NHPs (polyphenols, omega-3 fatty acids, and dietary fiber) that increase transcriptional control of mitochondrial biogenesis and indirectly improve metabolic flexibility.

9.5.1 Metformin and Berberine

Metformin is used as a first-line drug therapy for T2D and pre-diabetes. Its discovery dates to the 17th century when a traditional European plant, *Galega officinalis*, was used to relieve intense urination caused by T2D. The naturally occurring guanides in *G. officinalis* inspired the synthesis of metformin, one of three biguanide compounds to be synthesized in 1922.[56] Its mechanism of action continues to be defined but there is strong evidence for its action in the liver where it accumulates in mitochondria and inhibits complex I of the electron transport chain. This inhibition leads to decreased ATP levels, activated AMPK, and ultimately inhibition of fat synthesis and gluconeogenesis.[57] Another possible mechanism by which metformin inhibits liver-specific metabolism is by inhibiting glycerophosphate dehydrogenase, which is an important part of the glycerophosphate shuttle that moves reducing equivalents (*i.e.*, NADH) into mitochondria.[57] New research is showing that metformin may also inhibit mitochondria complex I activity in skeletal muscle isolated from obese and T2D human participants, which also showed activated AMPK leading to improved insulin action, increased glucose uptake, and a shift in cellular metabolism towards glycolysis.[58] Animal studies have also shown that oral dosing of metformin (at doses translatable to humans) predominantly leads to its accumulation in the liver

and intestines.[57] Other studies have discovered novel mechanisms by which metformin increases glucose utilization in the gut by increasing secretion of glucagon-like peptide-1 (GLP-1)[59] and causing significant modifications in the gut microbiome.[60] Clinical trials have shown that metformin has both preventative and therapeutic value in T2D. In a large clinical trial of 3234 non-diabetic patients, who were assigned to either placebo, metformin, or a lifestyle intervention,[61] a 2-year follow-up showed that both metformin and altered lifestyle reduced the occurrence of diabetes compared to the placebo.[61] Another large clinical trial in recently diagnosed diabetic patients showed that metformin could decrease glycemia, although not to normal levels, and not as well as insulin therapy.[62]

Berberine is a naturally occurring alkaloid compound (from *Berberis Vulgaris*) traditionally used in Chinese and Ayurvedic medicine to treat both heart disease and obesity. It is currently an approved NHP for the treatment of hyperlipidemia in several countries due to its cholesterol-lowering effects in humans.[63] Although large-scale clinical trials have not been conducted, berberine has been reported to be equally efficacious as metformin for its glucose-lowering effect in T2D patients.[64] Much like metformin, berberine's mechanism of action is thought be mediated through inhibition of liver-specific mitochondria complex I.[65,66] Interestingly, berberine was recently shown to have effects in the liver of obese mice where it inhibited FAO, restored PDH activity (improved glucose oxidation), and reduced pyruvate carboxylase activity (reduced gluconeogenesis).[67] In addition, strong preclinical evidence exists for berberine's thermogenic effects (increase of energy expenditure by mitochondrial uncoupling and biogenesis) in white and brown adipose tissues of obese mice.[68] However, unlike metformin, the effects of berberine in skeletal muscle of obese animals or humans are not well understood. Finally, its beneficial effects in heart disease are predominantly attributable to its lipid-lowering effects in the liver;[64–66] however, recent *in vitro* evidence shows its ability to inhibit FAO in cardiomyocytes under lipotoxic conditions, sparing glucose oxidation, and highlighting berberine's potential as a metabolic modulator.[69]

9.5.2 L-Carnitine

L-Carnitine is a water-soluble small molecule commonly found in a variety of foods but most concentrated in meat products such as beef.[70] It is sold as an ergogenic or weight loss dietary supplement and is traditionally associated with the transport of long-chain FFAs from the cytosol into the mitochondria for β-oxidation. L-Carnitine is essential in this role as neither long-chain FFAs nor their CoA esters can cross the inner mitochondrial membrane on their own (*i.e.*, transport is only possible when they are converted into carnitine esters). Beyond this classical role, it also impacts the acyl-CoA : CoA ratio whereby carnitine acyltransferase (CrAT) converts acetyl-CoA into its membrane permeant acetylcarnitine ester.[71] In animal models of obesity, the ability of L-carnitine and CrAT to augment

mitochondrial and intracellular carbon trafficking has been shown to combat nutrient stress, increase metabolic flexibility and enhance insulin action by permitting mitochondrial efflux of long-chain acetyl carnitines (from excess incomplete β-oxidation) that would otherwise impede glucose disposal through PDH.[14,72,73] While there is conflicting evidence on whether plasma L-carnitine levels drop or remain the same in T2D patients, several small-scale clinical trials have shown positive effects of L-carnitine supplementation on improving whole-body glucose oxidation and metabolic flexibility as well as reducing lipid parameters and oxidative stress markers in T2D patients.[73] Larger-scale clinical studies will determine if L-carnitine supplementation can be used as an adjuvant therapy for the treatment of T2D; however, more preclinical and clinical studies are warranted to determine its potential in obese and pre-diabetic individuals.

9.5.3 *R*-Lipoic Acid

As reviewed earlier, excessive FAO can activate PDK which allosterically inhibits PDH complex activity. Inhibition of PDH activity has been causally associated with metabolic inflexibility in obese and T2D patients. Earlier studies have evaluated the utility of dichloroacetate (DCA), a well-characterized pyruvate mimetic that inhibits PDKs by binding to the allosteric sites of PDK1, 2 and 4.[74,75] Treatment with DCA has been shown to improve glucose metabolism in animal models of diabetes and ischemia/heart failure;[76] however, its clinical translation was halted due to suboptimal pharmacokinetics, extremely low potency, reports of carcinogenic effects and other side-effects upon long-term exposure.[77,78]

R-lipoic acid (known as thioctic acid) is found in several vegetables and organ meat[79] and is a natural cofactor in mitochondrial enzyme complexes (*e.g.*, PDH and alpha-ketoglutarate dehydrogenase) that catalyze the oxidative decarboxylation of keto acids. Classically, the free form of lipoic acid is known to function as a potent water-soluble antioxidant by acting as a free-radical scavenger and by interacting with other cellular redox molecules, such as glutathione and vitamin E, to eliminate ROS.[80] Lipoic acid possesses a chiral carbon and can exist as either the *R*-(+) or *S*-(−) enantiomer where the *R*-(+) form is naturally occurring and possesses the greatest redox potential and ability to inhibit PDK and stimulate PDH activity.[81] In animal models of obesity and diabetes, chronic treatment with *R*-lipoic acid elicits improvements in whole-body glucose tolerance and insulin sensitivity.[80] It enhances insulin-stimulated skeletal muscle glucose transport and metabolism, which helps to ameliorate hyperinsulinemia, dyslipidemia, and oxidative stress.[80] Although evidence from large-scale clinical trials is currently not available, smaller clinical studies in T2D patients have reported improvements in whole-body insulin sensitivity and glucose disposal.[82–84] However, larger-scale trials on *R*-lipoic acid have recently been registered on clinicaltrials.gov to assess its therapeutic potential for the treatment of insulin resistance, pre-diabetes and T2D.

9.5.4 Thiamine

Thiamine is a water soluble B vitamin (vitamin B1) and an essential micronutrient commonly found in yeast, cereal grains, beans, and nuts.[85] It is currently included in multivitamin supplements, which often deliver up to 100% of the daily recommended value,[85] and is also used as a stand-alone supplement to help prevent fatigue and improve recovery in athletes.[86] Thiamine acts as a coenzyme for PDH and the α-ketoglutarate dehydrogenase complex and is thus essential for carbohydrate and amino acid oxidation. In T2D patients and power athletes, supplementation has been indicated, as thiamine levels have been reported to drop below the normal range.[86]

Studies in diabetic rats have confirmed that thiamine improves glucose tolerance and vascular complications associated with diabetes (retinopathy, nephropathy, and dyslipidemia).[85] However, sufficient clinical evidence is lacking to strongly indicate thiamine for use in obese and T2D patients. A pilot clinical study suggests that oral supplementation (300 mg thiamine per day for 6 weeks) modestly decreased 2 h postprandial plasma glucose levels but had no effect on markers of insulin resistance and fasting hyperglycemia compared to baseline in patients with impaired glucose tolerance.[87] Another pilot study in T2D patients showed that supplementation (300 mg per day for 3 months) significantly reduced urinary albumin excretion (*i.e.*, a symptom of diabetic nephropathy) but had no effect on glycemic control, dyslipidemia or blood pressure compared to controls.[88] More recent clinical trials have also reported modest improvements in fasting blood glucose but not in direct markers of insulin resistance.[89–91]

9.5.5 Nicotinamide Riboside

As shown in Figure 9.3, lipotoxicity and metabolic inflexibility in muscle leads to an accumulation of products of incomplete mitochondrial FAO. One of these products is the reduced form of nicotinamide adenine dinucleotide (NADH), which depletes mitochondrial levels of the oxidized form (NAD^+). Low levels of mitochondrial NAD^+ (low NAD^+ : NADH ratio) alter the acetylation state of mitochondrial proteins, including PDH, rendering them inactive. Mitochondria contain NAD^+-dependent deacetylases, called sirtuins (Sirts), that prevent ROS generation and lipotoxicity-associated acetylation of mitochondrial proteins (including PDH and proteins in the electron transport system). As such, Sirts have an integral role in metabolic flexibility by modulating glucose and lipid metabolism.[92] In animal models of obesity, T2D, and aging, muscle and liver levels of mitochondrial Sirts have been shown to decline.[92,93]

Nicotinamide (NAM) is one of three NAD precursor vitamins that is largely made available for NAD^+ salvage. Supplementation with NAD^+ precursors like nicotinamide riboside (NR) (commonly found in cow's milk[94]) has been proposed to increase levels of mitochondrial NAD^+ and increase Sirt activity and enhance metabolic flexibility. Recent studies in animal models of

obesity, T2D, and heart disease have shown that NR supplementation vastly improves glucose utilization by promoting PDH activity and reducing mitochondrial ROS generation.[90] A very recent preliminary yet comprehensive clinical investigation on NR supplementation (2 g per day for 12 weeks) in obese, insulin-resistant, sedentary males with elevated blood lipids showed that although NR was well tolerated, it did not improve insulin sensitivity, endogenous glucose production, glucose disposal and oxidation, resting energy expenditure, lipolysis, oxidation of lipids (as well as total cholesterol and low-density lipoprotein), or body composition.[95] Similarly, Martens *et al.* demonstrated that NR supplementation (500 mg per day for 6 weeks) in healthy middle-aged and older adults did not alter total energy intake and expenditure, oxidative fuel source (carbohydrate *versus* fat), physical activity patterns, body weight and body mass index, percent body fat, and measures of glucose or insulin regulation compared to placebo.[96] Martens *et al.* did report significant elevation of NAD^+ concentrations in circulating peripheral blood mononuclear cells (PBMCs) and beneficial reductions in systolic blood pressure and aortic stiffness (indicators of improved cardiovascular health).[96]

While the clinical evidence is still preliminary, future clinical trials are warranted to further investigate (1) if NAD^+ stimulation is achievable from alternative sources of NAD precursors such as dietary niacin (vitamin B3), (2) if tissue accumulation (adipose and skeletal muscle) of NAD^+ from NR (or alternative sources) supplementation occurs, and (3) to delineate why improvements in markers of metabolic flexibility were not observed.

9.5.6 Avocatin B

Lipids are rarely found to act as mitochondrial metabolic modulators. The effects of omega-3 fatty acids in mitochondria are well reported; however, their modulatory effect on metabolism is secondary to their activation of nuclear transcriptional factors that stimulate mitochondrial biogenesis and capacity.[55] Surprisingly, avocado-derived lipids were discovered to impart modulatory activity in mitochondria. Seventeen to twenty-one carbon-chain polyhydroxylated fatty alcohols (PFAs) were first discovered in avocado seeds (*Persea americana* Mill.; Lauraceae) in 1969.[97,98] Avocado seed-derived PFAs are a group of lipids with a long aliphatic chain, having a terminal unsaturation of either an olefinic (alkene) or an acetylenic (alkyne) nature, and multiple hydroxyl groups on the opposing end. Avocado PFAs have also been discovered in appreciable amounts in avocado pulp (the edible portion of the avocado).[99,100] A mixture of PFAs called avocatin B (1 : 1 mixture of avocadene and avocadyne) was recently shown to possess novel anticancer activity by accumulating in mitochondria and selectively inducing apoptosis of leukemia and leukemia stem cells through FAO inhibition.[101] Furthermore, avocatin B reversed glucose intolerance and insulin resistance in obese mice on high-fat diets by inhibiting FAO and improving metabolic flexibility in skeletal muscle.[102] Mechanistically, avocatin B inhibited FAO and increased glucose oxidation in pancreatic β-islet cells, which improved GSIS in lipotoxic

conditions. Similarly, avocatin B inhibited FAO in skeletal muscle cells, restoring both glucose uptake and oxidation, which were impaired due to lipotoxicity. A small randomized, double-blind, placebo-controlled human trial was also conducted where spray-dried avocado pulp powder standardized to avocatin B was consumed by healthy participants once per day for 60 days. Avocatin B at doses up to 200 mg per day was well-tolerated and associated with only mild adverse effects like gastric discomfort.

9.6 Conclusion

Mitochondrial dysfunction in obesity and associated disorders translates to metabolic inflexibility impacting whole-body physiology. Indeed, reduction in energy intake and prescription of exercise regimens should be at the center of any treatment designed to combat metabolic inflexibility; however, such measures are inherently difficult to implement at a population level. Metabolic modulation is only warranted in the right context: namely obesity-associated ectopic fat accumulation that elevates FAO in skeletal muscle, liver, pancreas, and heart. Increased insulin action and glucose uptake is generally desirable in all tissue types, but careful consideration of the fate of glucose after uptake is imperative since glycolysis and glucose oxidation (*via* PDH) are distinct fates, with the former causing decreased ATP production and lowering basal metabolism. More clinical studies are needed to validate the current molecular mechanism of metabolic inflexibility in several key tissues prone to lipotoxicity and insulin resistance.

Currently, clinically available metabolic modulators to reverse metabolic inflexibility in heart disease and, to some extent, in T2D have confirmed that mitochondrial targets like CPT-1, PDH, and PDK are indeed responsive to pharmacotherapy. However, the limited availability and narrow safety profiles of these synthetic agents highlight a glaring need to find novel metabolic modulators that can help slow down the global rates of obesity and associated disorders. This review highlighted specific NHPs that show great potential to be developed as therapeutic metabolic modulators to treat metabolic inflexibility. Despite their potential, multiple sufficiently powered clinical studies are still needed to further confirm the efficacy of these products, both in isolation and in combination with standard drugs, before they can be considered as suitable therapies. An optimal combination of compounds that target adipose tissue dysfunction, ectopic lipid accumulation, and metabolic inflexibility in pancreas, liver, heart, and muscle have the potential to mimic exercise-mediated effects and facilitate the treatment of obesity and associated disorders.

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CHAPTER 10

Nutraceuticals in Neurodegenerative Diseases

VIRGINIA BOCCARDI, CLARA TINARELLI AND PATRIZIA MECOCCI*

Institute of Gerontology and Geriatrics, Department of Medicine, University of Perugia, Santa Maria della Misericordia Hospital, Perugia, Italy
*Email: patrizia.mecocci@unipg.it

10.1 Introduction

Neurodegenerative diseases (NDDs) is an umbrella term that includes a range of conditions that primarily affect the neurons in the human brain. NDDs are incurable and debilitating conditions that result in progressive degeneration and death of neurons and other nerve cells. These disorders cause problems with motor or cognitive functioning, and are called ataxias or dementias, respectively. They include Alzheimer's disease (AD) and other dementias, Parkinson's disease (PD), Huntington's disease, motor neuron disease, Creutzfeldt–Jakob disease, and multiple sclerosis. Dementias are responsible for the highest burden amongst NDDs, with Alzheimer's representing approximately 60–70% of dementia cases.[1] These diseases pose a primary health problem especially in the aging population.[2,3]

Progress has been made pertaining to the etiology and pathogenic mechanisms of NDDs; however, there are currently no effective measures to prevent or treat the physiopathological processes. The mechanisms underlying NDDs are complex and multifactorial; however, accumulating evidence suggests a few commonly shared pathways including accumulation of

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pathologic proteins, oxidative stress, and inflammation.[4] Mechanisms on degenerative pathophysiology are mutable, mostly influenced by environmental factors and behavioral determinants[5] such as diet.[6] For example, certain dietary compounds such as polyphenols and endogenous compounds have considerable positive effects on the oxidative and inflammatory mechanisms.[7,8] Thus, nutraceuticals have recently gained importance owing to their multifaceted effects. These food-based approaches are believed to target multiple pathways in a slow but more physiological manner without causing severe adverse effects. There is growing interest in the use of natural products or herbal medicines for the prevention and treatment of NDDs. In this chapter, we summarize current research advancements on this topic.

10.2 Neurodegenerative Diseases: Principal Pathogenetic Mechanisms

10.2.1 Protein Aggregation

Proteins and peptides are essential complex macromolecules. Three-dimensional protein structures play critical roles within the cell, therefore proper folding is necesssary to perform these functions. Proteins can be exposed to internal and external forces (such as protein–protein interactions, stresses, mutations, *etc.*) that can alter protein conformation and biological activity. Also, newly synthesized proteins may not fold correctly or properly folded proteins can spontaneously unfold. In both cases, proteins have a strong tendency to aggregate. Protein aggregates within the cell have toxic effects when they accumulate above a certain level and many NDDs are characterized by accumulation of these abnormal protein aggregates.[9] The aggregates usually consist of fibers containing misfolded protein with a sheet conformation. Pathological forms of proteins appear to spread through the brain in characteristic patterns [*e.g.*, amyloid beta (Aβ), tau (τ), and α-synuclein (α-syn)].[10,11] The specific mechanism of how these aggregates induce neuronal toxicity is still unclear and under debate.

AD is the most common disorder and is characterized by the atrophy of gray matter and accumulation of amyloid plaques, which consist of extracellular aggregates of Aβ,[12] and neurofibrillary tangles (NFTs).[13] Aβ is a cleavage product derived from the amyloid precursor protein (APP) by the activity of β and γ-secretase.[14] The same Aβ peptide also occurs in AD-related vascular deposits in a condition known as cerebral amyloid angiopathy (CAA).[15] NFTs represent neuronal cytoplasmic aggregates of abnormal τ-protein consisting of paired helical filaments that develop after (and presumably directly from) pre-tangles and correlate with neuronal degeneration.[9] From the transentorhinal cortex, NFT pathology spreads into the entorhinal region and then further into other regions in a distinct hierarchical sequence that is different from that for Aβ-plaques.[16] The multiple system atrophy (MSA) is characterized by glial α-syn aggregations in the form of glial cytoplasmic inclusions (Papp–Lantos bodies) while the

neuropathological hallmark lesions of Lewy Bodies disease (LBD) are α-syn aggregates in neuronal cells termed Lewy bodies (LB) or Lewy neurites (LN).[9] While the neuropathological hallmark lesions of LBD are typically LB/LN, other pathologies are highly prevalent in LBD: for example, Aβ depositions could be found in up to 85% of dementia caused by LBD.[17,18]

10.2.2 Oxidative Stress and Inflammation

It is well known that reactive oxygen species (ROS) are chemically reactive molecules involved in the pathogenesis of NDDs. They play an essential role in the control of normal cellular activities such as inflammation, cell survival, and stress responses. They are however involved in many age-related diseases such as cardiovascular disorders, muscle dysfunction, immunological diseases, and cancers.[19,20] Oxidative stress (OS) is a result of the imbalance between pro- and anti-oxidant levels[21] in favor of pro-oxidants, but the precise pathway through which OS leads to NDDs remain largely unknown. Neurons are particularly vulnerable to pro-oxidants or ''free radicals'' and the high vulnerability of the nervous system, including the brain, spinal cord, and peripheral nerves, to OS is due to the elevated bioenergetic and oxygen requirements of these tissues.[22] This high-energy demand and massive consumption of oxygen in the respiratory chain can stimulate mitochondrial ROS production (*e.g.*, through Fenton and Haber–Weiss reactions).[23] OS causes damage by lipid peroxidation of plasma and mitochondrial membranes or oxidation of structural or enzymatic proteins causing irreversible modifications of nucleic acids and protein tertiary structure.

Numerous studies have investigated the role of ROS in the exacerbation of NDD progression through oxidative damage and interaction with mitochondria.[24] Under physiological and well-balanced conditions, ROS generated from mitochondria, NADPH oxidase (Nox), and xanthine oxidase (XO) are neutralized by endogenous antioxidants.[25] During disease, redox balance can be disturbed by neural inflammation and/or abnormal mitochondrial function.[26] Aggregation of misfolded proteins can in turn trigger inflammatory responses in the brain, which induce marked ROS production and subsequent OS.[27] This condition could be the link between the aggregation of proteins and OS.[28] Aggregated, altered or misfolded proteins that accumulate during aging or as a cause or consequence of pathology are able to activate the innate immune responses (*i.e.*, through damage-associated molecular patterns; known as DAMPs).[29] The same damaged neurons may also exacerbate immune-mediated disease by the release of chemokines involved in inflammation, including chemokine (C–C motif) ligand 21(CCL21),[30] which binds to and activates C–C chemokine receptor type 7 (CCR7) present on T cells, macrophages and microglia.[31]

Neuron necrosis, following acute brain injury and neurotrophic infection, leads to the release of damaging chemicals such as glutamate, nitric oxide (NO) and ROS.[32] For example, disturbance in glutamate homeostasis

because of astrocyte damage leads to excitotoxicity and is observed in acute viral encephalomyelitis, AD, PD, and acute brain injury. Another example is the activation of macrophages and microglia during infection, trauma, following exposure to toxic compounds or, indeed, upon aging. Chronic microglia activation may compromise blood–brain barrier integrity and lead to an increased influx of peripheral immune cells rwre.

Considering the pivotal roles of OS in NDDs, the manipulation of ROS levels may represent a promising treatment option to slow down neurodegeneration and alleviate associated symptoms.

10.3 Nutraceuticals

The term ''nutraceutical,'' derived from nutrition and pharmaceutical, can be defined as ''a food, or a part of it, that provides medical or health benefits, including the prevention or treatment of a disease.'' Several nutraceuticals can act as protective agents in NDDs. Historically, phytochemical therapies have been used against neural symptoms; however, the underlying mechanism of action of these treatments are yet to be determined. Nonetheless, evidence supports their potential efficacy to act as anti-oxidant and anti-inflammatory agents with particular activity in altering neuronal excitability *via* inhibiting or activating ion channels or specific receptors ewrw. Several neuroprotective mechanisms of nutraceuticals seem to proceed *via* suppression of lipid peroxidation, inhibition of inflammatory mediators, modulation of gene expressions, and activation of anti-oxidant enzymes, which make them ideal therapeutic options for the treatment of NDDs. The major classes of phytochemicals are flavonoids and non-flavonoid polyphenols. Flavonoids include flavanols, flavonols, flavones, isoflavones, and anthocyanidins. Non-flavonoids include resveratrol and curcumin. Other phytochemicals include carotenoids, crocin, B vitamins, and diterpenes. The most studied nutraceuticals and their application in NDDs are reported below.

10.3.1 Flavonoids

Flavonoids (Figure 10.1) are a group of polyphenolic compounds that are very common in most plants, including fruits, vegetables, and several types of natural drinks such as tea, cocoa and wine.[33] Over 9000 flavonoids have been identified and are characterized by their chemical structure, which can be further divided into six subgroups based on their tertiary structure. Flavonoids and their metabolites exert countless health-promoting effects. They have been shown to have an impact on multiple biological processes such as anti-viral, anti-allergic, anti-platelet, anti-inflammatory, anti-tumor, and anti-oxidant activities.[34] They can also modulate several neurological processes including modification of the neuronal-glial signaling pathways involved in neuronal survival and function, inducing changes in cerebral blood flow, upregulating anti-oxidant enzymes and proteins involved in

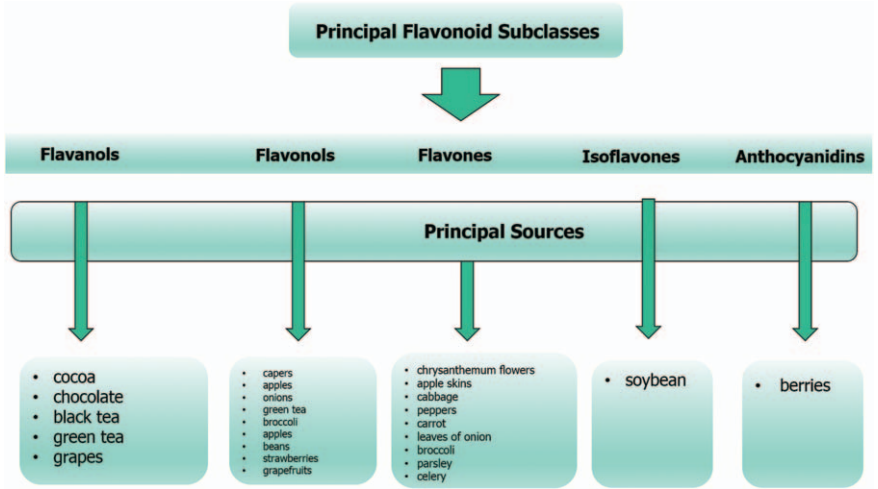

Figure 10.1 Schematic representation of the main flavonoid subclasses and sources.

synaptic plasticity and neuronal repair,[35] and inhibiting processes typically affected in AD pathogenesis.[36] Moreover, they can cross the blood–brain barrier (BBB) and may exhibit their functions directly at the molecular level influencing protein function and gene expression. For example, flavonoids have been shown to upregulate brain-derived neurotrophic factor (BDNF) and improve performance of spatial memory.[37] Several pieces of evidence have suggested their role in the attenuation of pathological pathways of NDDs.[38] Furthermore, flavonoids can modulate the neuronal immune system and attenuate neuroinflammation by inhibiting the production of NO and cytokines induced by activated microglia.[39]

10.3.1.1 Flavanols: Catechin, Epicatechin, Epigallocatechin Gallate

Flavanols are a flavonoid group and are found in cocoa, chocolate, black and green teas, and grapes. Epidemiological observations in Finnish and US populations showed a reduced risk of PD in high consumers of tea;[40] a similar negative association between cognitive impairment and green tea consumption was found in a Japanese population.[41] Research over the past decade has identified flavanols as showing diverse beneficial physiologic and anti-oxidant effects, particularly in the context of vascular function.[42]

Catechin and epicatechin are the most abundant flavanols in grape seeds and grape juice. A study showed that supplementation with grape juice from a variety of *Vitis vinifera* called Koshu, inhibited glutamate excitotoxicity,[43] which is implicated widely in the pathogenesis of a range of neurological diseases, including AD, Huntington's disease and traumatic brain injury.

Additional human studies with grape juice have shown that short- and medium-term supplementation produces benefits in individuals with cerebrovascular diseases including increased serum antioxidant capacity, reduced lipid peroxidation, improvement of endothelial function, and reduction of platelet aggregation.[44]

Epigallocatechin gallate (EGCG) is a type of catechin and the major constituent of green tea responsible for its health-promoting potential. Polyphenols from green tea and in particular EGCG have been reported to exert anti-oxidant,[45] anti-carcinogenic,[46] and anti-inflammatory effects.[47] For example, daily EGCG treatment in rats has been demonstrated to reduce the progressive increase of OS induced by hypertension. The anti-oxidant activity can attenuate neurotoxicity and prevent neuronal damage and hippocampal neuronal cell death resulting from ROS-induced damage.[48] Interestingly, EGCG obstructs the augmented caspase activity induced by Aβ, which leads to neuronal cell death.[46] Moreover, it also attenuates the hallmarks of AD pathology such as the interaction between ROS, apoptosis, and Aβ. Most importantly, animal model studies have shown that EGCG can cross the BBB and can reach the brain parenchyma.[47]

Consumption of cocoa flavanols has been previously shown to influence cerebral hemodynamics. It has been suggested that one consequence of the effect of cocoa flavanols on cerebral blood flow might be to improve performance on visual and cognitive tasks, as was shown after consumption of cocoa beverages.[49] Recent evidence is also showing that extracts from tea, especially catechins, are primarily involved in the prevention of pathological aggregation of abnormal proteins.[50]

10.3.1.2 Flavonols: Quercetin, Kaempferol

Quercetin is one of the most studied flavonols, commonly found in capers, apples, onions, and green tea. It is also present in medicinal plants such as *Sambucus canadensis*, *Hypericum perforatum*, and Ginkgo biloba (Gb).[51] Standardized extracts of Gb leaves are studied for their potential to improve memory and cognitive function. Hemodynamic, neurotransmitter, and free-radical scavenging effects of Gb have been shown in several studies. All of these biological functions may be relevant to aging and age-related disorders.[52] The primary activity of quercetin is to prevent endothelial apoptosis caused by pro-oxidants, as it possesses highly potent anti-oxidant activity.[3] Furthermore, quercetin significantly protects neuronal cells from neurotoxicity induced by OS.[53] Indeed, *in vitro* studies have shown that quercetin increases cell survival in neurotoxic conditions (*i.e.*, in the presence of hydrogen peroxide, linoleic acid hydro-peroxide, and interleukin-1β).[50] Pre-clinical studies have shown that quercetin could play a role in vascular dementia by decreasing the size of ischemic lesions. It has been shown to inhibit or stimulate signal transduction pathways that have positive effects on the central nervous system (CNS) including improvements in cognition and imparting anti-anxiety effects.[54] Quercetin in AD attenuates

Aβ aggregation and decreases levels of the enzyme beta-secretase 1 (BACE1), which mediates the cleavage of APP.[55]

Kaempferol is a phytoestrogen found in tea, broccoli, apples, beans, strawberries, and grapefruits.[56] It protect PC12 cells (a cell line derived from a pheochromocytoma of the rat adrenal medulla, which has embryonic origin and is a mixture of neuroblastic cells and eosinophilic cells) against OS induced by hydrogen peroxide (H_2O_2) and has been shown to improve cognitive learning and memory capability in mice.[56,57] Anti-inflammatory and anti-oxidative effects have been noted[58] with efficient neuroprotective effects against necrosis and apoptosis-inducing damaging agents.[59–61]

10.3.1.3 Flavones: Luteolin, Apigenin

Luteolin is found in fruits and vegetables, such as chrysanthemum flowers, apple skins, cabbage, peppers, carrot, and leaves of onion, broccoli, parsley, and celery and is the most abundant flavonoid – a subclass of the flavones. It shows potential anti-inflammatory and anti-oxidative properties and exhibits phytoestrogen like activities.[62] Phytoestrogens operate primarily through binding to estrogen receptors (ERs) and exert a wide range of beneficial effects on the cardiovascular, metabolic, and central nervous systems. Moreover, it has been shown that luteolin has several pharmacological properties including a protective role against DNA damage, and hydrogen peroxide-induced toxicity, and anti-inflammatory actions.[63] Recent animal studies have found that AD pathologies in mice, caused by traumatic brain injury, can be reduced by luteolin (20 mg kg^{-1} per day for 15 days).[64] Aβ generation was also lessened when luteolin was given to primary neuronal cells from SweAPP-overexpressing mice (an animal model with onset of AD from 3–15 months of age associated with age-dependent oligomeric Aβ).[65] Luteolin administration also protected hippocampus structure and prevented learning flaws in a astreptozotocin-stimulated AD rat model.[66]

Apigenin can be found in common vegetables and fruits such as onion, parsley, grapefruit, and orange.[67] It exhibits multiple pharmacological effects such as anti-inflammatory, anti-apoptotic, anti-oxidative, purgative, anti-viral, and anti-mutagenic.[68] It has been shown to reduce glutamate-induced calcium signaling in murine cortical neurons[69] and protect neurons against Aβ-mediated toxicity in AD by dissipating mitochondrial membrane and neuronal nuclear condensation.[70] It can modulate GABA(gamma-aminobutyric acid)ergic and glutamatergic transmission in cultured cortical neurons, improve memory impairment associated with AD, prevent OS, and decrease the burden of Aβ plaques. Numerous studies have demonstrated anti-inflammatory[71] and anti-apoptotic effects in various animal models as well as in humans.[72] It has been shown that administration of 10–100 μM apigenin can reduce NO and pro-inflammatory cytokine production while improving the cholinergic signal by acetylcholinesterase inhibition in NDDs.[73]

10.3.1.4 Isoflavones

Isoflavones are primarily found in soybeans (soy).[74] They can improve cognitive function by mimicking the effects of estrogen, in particular through binding to estrogen receptor β in the brain.[75] Soy isoflavones are considered agonists of ERs and estrogen-replacement therapy can improve cholinergic function by increasing choline uptake and potassium-stimulated acetylcholine release.[76]

Evidence has shown that soy isoflavones may improve visual-spatial memory, learning ability, and memory.[77] A study on young and mature mice demonstrated that soy-supplementation decreases thiobarbituric acid reactive substances and increases plasma glutathione (GSH) peroxidase levels.[78] Dietary soybean isoflavones contain multiple phenolic hydroxyls that react with and neutralize free radicals, thus playing a role in inhibiting lipid peroxidation and increasing the activity of anti-oxidant enzymes.[79] In animal studies, soybean isoflavones alone or in combination with folic acid have been demonstrated to significantly improve total anti-oxidant capacity, GSH, and GSH-peroxidase levels in brain tissues and blood.[80] While the exact mechanisms are under investigation, soy isoflavones protect nerve cells by also upregulating anti-apoptotic genes while downregulating pro-apoptotic genes.[81] Further evidence suggests that high intake of isoflavones upregulates circulating blood vascular endothelial growth factor and NO levels while downregulating von Willebrand factor, thus protecting against vascular endothelial damage and preventing neuronal regression.[82]

10.3.1.5 Anthocyanidins: Cyanidin-3-glucoside, Pelargonidin

Anthocyanidins are common plant pigments. They are the sugar-free counterparts of anthocyanins, which are flavonoids found in berries. There is strong evidence suggesting that blueberry anthocyanins can improve memory and learning in aged animals, which is linked to modulation of structural and synaptic plasticity markers, such as ERK, cAMP-response element-binding protein (CREB), and BDNF.[83] A potential role of anthocyanins in neuroprotection is mediated through phospholipase A2 inhibition,[84] which is negatively involved in pathways linking receptor agonists, oxidants, and pro-inflammatory cytokines. Neurogenesis in the hippocampus is another mechanism by which blueberry flavonoids may improve memory.[3] Ingestion of berries has been shown to modulate the expression of particular proteins like CREB, which is a pathway activated in response to Aβ and BDNF. Changes of these two proteins in berry-fed animals were accompanied by increases in the phosphorylation state of protein factors crucial for synaptic plasticity and memory formation.[85]

Cyanidin-3-glucoside (C3G) extracted from mulberry has been shown to impart neuroprotective properties against nutrient depletion (*e.g.*, glutamate, oxygen, and glucose)-induced neuronal cell death.[86] C3G can neutralize the level of Aβ1–42 peptides and minimize H_2O_2-induced neurotoxicity.[87]

More recently, it has also been shown that C3G significantly attenuates Aβ25–35-induced expression of ER stress proteins in neuronal cells.[88]

Another study that highlights the protective role of berries demonstrated that berry extracts decrease intracellular calcium activity and ATP leakage, leading to reduced aggregated Aβ.[89] Animal studies confirm anti-oxidant activities by the alteration of ROS signaling through CREB and MAP-kinase.[90] Finally, there is evidence that anthocyanins have insulin-like and glitazone-like properties, which may contribute to improved metabolic function and lipid-lowering effects as well as improved memory and a reduction of depressive symptoms.[91] In humans, a prospective evaluation with a food-frequency questionnaire showed that a greater intake of blueberries and strawberries was associated with lower rates of cognitive decline in subjects older than seventy years, suggesting a potential protective role of berries in cognitive function.[92]

Pelargonidin (Pel) is a flavonoid found in orange and red colored fruits that is efficiently absorbed in the gastrointestinal tract and is able to cross the BBB. It is an ER agonist[93] and exerts a vast number of beneficial effects. It possesses anti-oxidant,[94] anti-hyperglycemic,[95] and anti-thrombosis activity and protects neurons from DNA damage. In addition, it modulates interleukin-10 (IL-10), which contributes to the protective effects against inflammatory disease and has been shown to delay age-related memory and cognitive deficits.[93] As an anti-oxidant, Pel inhibits the inducible nitric oxide synthase (iNOS) protein, NO production, and NF-κB expression.[96] Finally, studies have shown that oral Pel consumption (10 mg kg) prevents memory loss induced by Aβ *via* ER-independent pathways.

10.3.2 Non-flavonoid Polyphenols: Resveratrol and Curcumin

Resveratrol is a polyphenol found in seeds and skin of several fruits such as grapes. It has been shown to increase 5-hydroxytryptamine (5-HT) activity, which supports its anti-depressant properties.[3] In fact, animal studies have shown that resveratrol inhibits the re-uptake of noradrenalin and increases 5-HT, leading to an increase of hippocampal serotonin levels. As an anti-oxidant, resveratrol is also able to reduce inflammation and protect neurons from oxidation-induced neuronal toxicity.[97] There is also a reverse association between dementia and subjects drinking moderate amounts of red wine. A possible mechanism for the neuroprotective action, especially in AD, is its capacity to modulate Aβ processing.[98] New evidence has shown that resveratrol can markedly decrease levels of secreted and intracellular Aβ peptides by facilitating Aβ proteolytic clearance in different cell lines and primary neurons.[99] Finally, it has been shown to be able to modulate autophagy and new evidence underlines how the activation of autophagy, a pathway that degrades and recycles macromolecules, may be useful in NDD prevention or treatment.[100]

Curcumin is the most active element of turmeric (*Curcuma longa*), an herb of the ginger family. A diet rich in curcumin may reduce the risk of NDDs, including AD. The prevalence of AD in India, where curcumin is a staple spice, is much lower than in other countries.[101] Curcumin may act through

different mechanisms such as anti-oxidant, anti-inflammatory, and anti-amyloidogenic (*i.e.*, preventing plaque formation). While the exact mechanism remains unclear, its ability to reduce depression is linked to inhibiting monoamine oxidase levels, which modulates serotonergic, dopaminergic, and noradrenergic transmission.[102] In mouse models of encephalitis, curcumin decreases ROS and inhibits pro-apoptotic signals in neuronal cells.[103] It also reduces Aβ-related cerebral burden and inflammation in transgenic AD mice.[104]

10.3.3 Carotenoids

There are over 700 different carotenoids, which include lycopene, lutein, zeaxanthin, β-cryptoxanthin, α-carotene and, the most prominent, β-carotene.[104] They are found mostly in red, orange, and deep yellow vegetables and fruits and also in seafood.[105] Carotenoids may inhibit the onset of NDDs through a variety of mechanisms, which include anti-oxidant capacity through ROS quenching, upregulation of anti-oxidant enzyme systems, hypocholesterolemic properties, anti-neuroinflammatory effects, anti-amyloid aggregation activity, and regulation of amyloid oligomer-induced signaling.[106,107] Carotenoids may ameliorate mitochondrial dysfunction, OS, sustained neuroinflammation, impaired lipid metabolism, Aβ aggregation, and Aβ neurotoxicity,[108] which are all activities associated with AD pathogenesis.[109,110] Lycopene administration has been shown to reduce Aβ-induced mitochondrial dysfunction, inflammatory cytokine release, and caspase-3 activity in an AD rat model.[111] Furthermore, astaxanthin treatment reduced Aβ-induced damage in a cell model system through several mechanisms including downregulation of apoptotic factors, inhibition of pro-inflammatory cytokine activity, and ROS reduction.[112]

10.3.4 Crocin (Saffron)

Crocin, extracted from saffron, has been traditionally used as an anti-spasmodic, gingival sedative, carminative, and expectorant stimulant compound.[113,114] It has been more recently reported to act as an anti-convulsant, anti-depressant, and anti-inflammatory element.[115] Crocin has also been shown to improve learning and memory and prevent ethanol-induced long-term potentiation block in mice.[116,117] In human subjects with mild-to-moderate AD, it improved cognition as evaluated using neuropsychological scales.[118] Furthermore, it demonstrates anti-oxidant properties by significantly modulating levels of oxidative markers in the hippocampus.[119] Interestingly, the observation that the anti-oxidant effects of saffron extract and purified crocetin are significantly potentiated by the presence of living cells[120] suggests either that additional cell-mediated metabolism is required to enhance saffron's direct scavenging capacity or that saffron acts by further stimulating cells to upregulate their endogenous anti-oxidant defenses. These hypotheses are now under investigation.[121] One mechanism by which

endogenous defenses might be activated is in response to stress, which raises the critical question of whether saffron, like other phytochemicals,[122] confers its neuroprotective effects by acting as a mild stressor to condition cells against more severe insults.

10.3.5 B-vitamins: Folate, Cobalamin, Pyridoxin

Folate (vitamin B9), cobalamin (vitamin B12), and pyridoxin (vitamin B6) are the most important B-vitamins in the field of cognition[3] and are essential for maintaining the integrity of the nervous and hematopoietic systems. B-vitamin deficiency, for example, can result in irreversible neurological disorders such as peripheral neuropathy.[123] Folate and cobalamine deficiencies cause accumulation of homocysteine, which is generally linked to NDD. Many studies have found associations between low circulating folate levels or hyperhomocysteinemia and low cognitive scores, such as Mini Mental State Examination (MMSE) scores.[124] Vitamin B12 is involved in the methylation of homocysteine to methionine for the synthesis of methyl acceptors such as membrane phospholipids, myelin, and neurotransmitters. In the brain, hyperhomocysteinemia is not only negatively correlated with neuropsychological tests scores but it is also considered a marker for low serum vitamin B12 and folate.[125] Poor vitamin B6 status is common among older people, and it is involved in the regulation of mental function and mood. It is also an essential homocysteine re-methylation cofactor, and its deficiency is associated with an increase in blood homocysteine levels; it is also potentially toxic to neurons, as its levels are associated with atrophic changes.[126]

Treatment with vitamin B12 or B6 has been demonstrated to stabilize performance on attention, executive and planning function.[127] In addition, B6 vitamin concentration has been associated with better global cognition scores, especially with better scores in attentive and executive functions.[128] However, and in contrast, a recent Cochrane Review concluded that there is no clear evidence that short-term treatment with vitamin B6 improves mood (depression, fatigue, and tension symptoms) or cognition.[129] Further, recent randomised controlled trials (RCTs) on the effects of folate, vitamin B12, and vitamin B6 failed to show any significant effect.[130] Therefore, it remains to be established if prolonged treatment with B-vitamins can reduce the risk of dementia in later life.

10.3.6 Diterpenes: Carnosic and Rosmarinic Acids

Carnosic and rosmarinic acids (RA) are part of the family of phenolic acids and diterpenes and are two of the most important anti-oxidant compounds in the herb rosemary.[131] These compounds have shown neuroprotective action in cell culture models of neuronal death and in pre-clinical neurodegeneration models. They scavenge reactive nitrogen species and have been shown to protect neuroblastoma cells from hydrogen peroxide-induced OS.[132] In AD, diterpenes significantly alleviate memory impairment

associated with Aβ neurotoxicity.[133] The inhibition of β-sheet formation and assembly by RA has also been documented.[134] Hence, both extracellular (Aβ) and intracellular (tau) protein aggregation, which are clinically seen as AD pathological markers, are targeted by RA.[135] Another mechanism might be explained by the recently identified role of angiogenesis in the formation of β-amyloid plaques. Although the mechanism is unclear, carnosic acid has demonstrated antiangiogenic properties.[136]

10.4 Conclusions

Abnormal protein aggregation, OS, and inflammation are common pathways involved in the neuronal degeneration leading to NDDs. Changes in nutrition may play an essential role in brain and major organ function during aging. Susceptibility of the older population to specific nutrient deficiencies may exacerbate some processes of cognitive decline and NDDs. There is a large amount of evidence on the actions of several nutrients on NDDs and supporing evidence on the potential role of nutritional supplementation to prevent age-related cognitive decline and neurodegeneration. Several components of phytochemicals have been shown to have benefits for these diseases. Unfortunately, there is a lack of well-conducted studies and standardized human RCTs, which limits the ability to draw significant and strong conclusions. In addition, very few studies test the effects of combinations of more than one of these substances, which reflects the true nature of supplements. In the future, stronger scientific evidence should confirm this extremely promising program of treatments against NDDs.

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CHAPTER 11

Probiotic Supplements – Basic Concepts of the Gut Microbiome and the Role of Probiotics to Sustain Health

ASMAA ALRAEFAEI,[a] AMEL TAIBI[a] AND ELENA M. COMELLI*[a,b]

[a] Department of Nutritional Sciences, Faculty of Medicine, University of Toronto, 5th Floor, Medical Sciences Building, 1 King's College Circle, Toronto, Ontario M5S 1A8, Canada; [b] Joannah and Brian Lawson Centre for Child Nutrition, Faculty of Medicine, University of Toronto, 5th Floor, Medical Sciences Building, 1 King's College Circle, Toronto, Ontario M5S 1A8, Canada
*Email: elena.comelli@utoronto.ca

11.1 Introduction

Were probiotics largely responsible for maintaining human health through unforgiving historical times? While it may be scientifically challenging to provide a definitive answer to this question, it certainly provokes intriguing thought on our ancestral wisdom. For centuries, traditional diets from various regions across the world consisted of fermented staple foods, notably fermented dairy produced from raw, mammalian milk.[1] Only in the past century has modern science appreciated such wisdom when investigations were conducted on the important contributions of the microorganisms responsible for food fermentation to maintaining human health.[2] Although

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many fermented foods contain microorganisms genetically similar to probiotic strains,[3] they often contain dead and/or undefined microorganisms and are thus not classified as probiotics.[4] By definition, probiotics are ''live microorganisms that, when administered in adequate amounts, confer a health benefit on the host''[5,6] and are commonly isolated from the intestinal contents or feces of humans or animals, from human breast milk, or from foods such as raw milk, fermented dairy, or non-dairy products.[7] Probiotics are found in foods such as dairy products (yogurt, drinkable yogurt, fermented milk/kefir, cheese), infant formula, fermented foods labeled as being probiotic, cereals, and juices, or sold as supplements (including capsules or sachets of powder).[8,9] This chapter focuses on probiotics as nutraceutical supplements.

A close interplay exists between nutraceuticals and the gut microbiome in which the administration of nutraceuticals such as probiotics, prebiotics, phytoestrogens, polyphenols, algae, and curcuminoids, among others, has been demonstrated to modulate the gut microbiome composition to reflect a more health-compatible one.[10] The gut microbiome is a thrilling new field especially in the context of precision medicine due to its potential role in impacting health and disease risk at the single patient level.[11] The issue of whether the gut microbiome is simply a matter of correlation with health and disease or causation remains particularly elusive in humans.[12] Nonetheless, recent evidence from studies investigating humans and subsequent findings in mice has indicated a causal role for the gut microbiome in obesity,[13] childhood asthma,[14] and irritable bowel syndrome (IBS).[15]

The gut microbiome spans the entire intestinal ecosystem, including resident microorganisms, their genomes, and the environmental conditions.[16] With pivotal advances in our understanding of how the human gut microbiome is impacted, probiotics are considered a dietary strategy to modulate the gut ecosystem to potentially prevent and treat chronic diseases early and later in life.[17,18] This therefore situates probiotics at the forefront of gut microbiome-targeted therapies to ultimately ease the burdens of chronic diseases that have afflicted our public health for far too long. This chapter discusses basic concepts of the gut microbiome, benefits of probiotics, mechanisms of their activity, and provides fundamentals to understand the inclusion of probiotics in nutraceuticals.

11.2 Gut Microbiome

11.2.1 Definition and Functions of the Gut Microbiome

Described as ''Worlds within worlds'',[19] regarded as a forgotten, hidden organ with a collective metabolic activity equal to an additional organ within us,[20] and estimated to marginally surpass human cells in a ratio of 1.3 bacterial cells to every human cell,[21] it comes as no surprise that the gut microbiome has garnered incredible volumes of interest from scientists

around the globe. By definition, the gut microbiome ''refers to the entire habitat, including the microorganisms (bacteria, archaea, lower and higher eukaryotes, and viruses), their genomes (*i.e.*, genes), and the surrounding environmental conditions''.[16] A pioneering study on the human gut metagenome established a non-redundant human gut microbial gene catalog using fecal DNA from 124 European adults of Nordic and Mediterranean origins.[22] The catalog consisted of 99.1% bacterial, ~0.8% archaeal, and 0.1% eukaryotic and viral genes. Moreover, 38% of the genes from each individual were shared with at least half of the individuals of the cohort, indicating a common microbial gene pool. A selection of the top functional complementarities (expression coherence index value $\geq$20) between the human genome and gut metagenome include the metabolism of purines and pyrimidines, glycine, serine, and threonine, glycolysis/gluconeogenesis, glutamate, fructose and mannose, aminosugars, and starch and sucrose.

The functions of the gut microbiome are unequivocally essential to host development and physiology and are exerted proximally in the intestines and distally at various body sites. Some of these functions include fermentation of indigestible polysaccharides into utilizable metabolites such as short-chain fatty acids (SCFAs), synthesis of essential vitamins, protection from opportunistic pathogens, maturation and differentiation of the immune system, promoting the strength and integrity of the intestinal barrier, and influencing metabolism, adiposity, brain, and behavior.[23] Interestingly, studies comparing germ-free mice to conventional specific-pathogen-free mice have demonstrated the magnitude of the impact that the gut microbiome exerts on distal body sites, including sites as encased and as concealed as the brain[24] and bone marrow,[25] suggesting its essential role in regulatory processes not limited to the intestines.

11.2.2 Establishment, Development, Composition, and Factors Influencing the Gut Microbiome

When, during development, is the human gut microbiome established has been a question of immense debate amongst scientists.[26,27] The question refers to whether the gut microbiome is established *in utero* or if this process commences during birth? Evidence in the past decade or so is suggestive that this process is initiated *in utero* not only in humans, but rather, is considered a universal phenomenon observed in the animal kingdom.[26] In humans, evidence for this is presented in studies that have detected non-pathogenic microorganisms in fetal membranes,[28] umbilical cord blood[29] and amniotic fluid[30,31] from healthy, full-term neonates and placentae[30–32] from healthy mothers with no infection or inflammation.

Studies in distinct populations from Venezuela, Malawi, USA, and Sweden collectively suggest that the gut microbiome stabilizes between 1 and 3 years

of age.[33,34] This notwithstanding, compositional and functional variations of the gut microbiome between adults and pre-adolescent children 7–12 years of age have been described[35] suggesting that the gut microbiome may continue development beyond early childhood.

The adult gut microbiome is primarily composed of species from two dominant phyla, Firmicutes [*e.g. Lactobacillus* and *Clostridium* species (spp.)] and *Bacteroidetes* (*e.g. Bacteroides* spp.). Other phyla include Proteobacteria (*e.g. Escherichia* and *Helicobacter* spp.), Actinobacteria (*e.g. Bifidobacterium* spp.), *Tenericutes* (*e.g. Mycoplasma* spp.), and Verrucomicrobia (*e.g. Akkermansia* spp.). It is crucial to note that healthy humans share more of a core microbiome from a functional perspective rather than from a compositional perspective, meaning that although high inter-individual variability of bacterial species exists, the genes and/or metabolic capabilities are shared across the majority of the gut microbiomes.[36,37]

A number of modifiable and non-modifiable factors influence the gut microbiome including maternal gut microbiome or diet or drugs, delivery and feeding modes, host genetics, diet, antibiotics and other drugs throughout life, infections, diurnal rhythm, environmental microbial exposures, sex, age, and exercise.[38,39] Together, these factors contribute to a eubiotic or dysbiotic microbiome with implications for health and disease. The concepts of eubiosis and dysbiosis are integral to understanding the role of the gut microbiome in homeostasis. Eubiosis is defined as ''the state of having a healthy population of bacteria naturally occurring in a body site'',[40] while dysbiosis refers to ''an imbalance in the structural and/or functional configuration of the microbiota, leading to a disruption of host–microorganism homeostasis''.[23] Examples of dysbiosis associated with intestinal and systemic diseases or conditions are obesity, metabolic syndrome, nonalcoholic steatohepatitis, inflammatory bowel diseases [Crohn's disease (CD), ulcerative colitis (UC), and pouchitis], IBS, atherosclerosis, type 1 diabetes, autism, allergy, asthma, and celiac disease, among others.[41] Probiotics supplementation may be a dietary strategy to prevent or minimize dysbiosis and/or to restore the gut microbiome.[40]

11.3 Probiotics

11.3.1 Benefits of Probiotics

In the consensus statement on the scope and appropriate use of the term probiotic published by the International Scientific Association for Probiotics and Prebiotics in 2014,[5] the expert panel agreed that sufficient scientific evidence is available to support the concept that certain probiotics confer core benefits. These core benefits can be derived from administering an adequate amount of any safe strain of a given species identified to be an effective probiotic.[5] To substantiate this concept, a meta-analysis of randomized controlled trials in humans investigating probiotic efficacy for gastrointestinal diseases[42] reported that 8 of 11 species and species mixtures

showed significant positive effects for the treatment and prevention of gastrointestinal diseases, suggesting core benefits for some broader classes of defined, live microbes. Examples of core benefits include supporting a healthy digestive tract and immune system, and are discussed below. On the other hand, species- or strain-specific benefits include synthesis of vitamins, enhancement of the intestinal barrier, metabolism of bile salts, immunomodulation, and neurology or endocrinology-related effects.[5] In 2015, an expert consensus opinion on recommendations for probiotic use was published, which assigned grades to probiotic effectiveness based on the degree of evidence available[43] (for a detailed description of the grading, refer to ref. 44). These recommendations are useful in clinical practice because they provide a basis for probiotic selection. Below, we discuss specific benefits drawn from this document and literature for infants and children and adults.

11.3.1.1 Benefits of Probiotics in Infants and Children

The benefits of probiotics in infants and children include prevention and treatment of a number of clinical conditions. Studies highlight that specific benefits are associated with specific probiotics species and strains. For example, *Lactobacillus* GG and *B. lactis* are effective for the treatment and prevention of atopic eczema associated with cow's milk allergy,[43,45] while *L. reuteri* 17938 can be considered for the treatment of infantile colic.[46] Benefits have also been found for a range of infectious diseases of the respiratory tract or the intestine. For example, various lactobacilli, alone or in combination with bifidobacteria, may be considered as a prophylactic strategy against upper and lower respiratory tract infections (reviewed in ref. 47). A meta-analysis of six randomized controlled trials ($n = 766$) found that probiotics (*Lactobacillus* GG, *S. boulardii*, or *B. lactis* and *S. thermophilus*) prevent antibiotic-associated diarrhea (AAD).[48] Probiotics have also been shown to prevent *Clostridioides difficile*-associated diarrhea (CDAD) and to treat infectious diarrhea, using *Lactobacillus* GG and *S. boulardii* for the former, and *Lactobacillus* GG, *S. boulardii*, and *L. reuteri* SD2112 for the latter.[43] For preterm or very low birth-weight infants, trials report a role of selected probiotics in preventing necrotizing enterocolitis,[49] but further substantiation is necessary. In terms of intestinal disorders, VSL#3, a probiotic mixture of eight bacterial species (*L. acidophilus*, *L. plantarum*, *L. paracasei*, *L. delbrueckii* subsp. *bulgaricus*, *B. breve*, *B. longum*, *B. infantis*, and *S. thermophilus*) is beneficial for the treatment of IBS symptoms in children[43] and has positive effects in active UC, while evidence remains inadequate for CD.[43,50]

11.3.1.2 Benefits of Probiotics in Adults

Similarly to children, the benefits of probiotics have been demonstrated for the prevention and treatment of various conditions in adults. Probiotics

from the *Lactobacillus* genus prevent AAD,[51] and various probiotic bacteria and yeast including *Lactobacillus GG* and *S. boulardii* prevent CDAD.[52] *B. infantis* 35624 has been shown to relieve IBS symptoms in women.[53] Benefits of probiotics in inflammatory bowel diseases, including pouchitis, UC, and CD, have been investigated in many trials. VSL#3 prevents and maintains remission of pouchitis[43,54] and *Escherichia coli* Nissle 1917 and VSL#3 maintain and induce remission of UC;[43,55,56] however, the benefits of probiotics for the management of CD currently remain unconvincing.[57] In terms of infection, a recent meta-analysis of 10 parallel controlled trials revealed that supplementation with *Lactobacillus*- and *Bifidobacterium*-containing probiotics along with *H. pylori* eradication therapy increased eradication rates and decreased total side effects.[58] Additional benefits include the use of *B. animalis* subsp. *lactis* for the management of occasional constipation, the use of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* for the management of lactose intolerance, the use of specific *Lactobacillus* and *Bifidobacterium* strains for reducing the incidence and duration of common infectious diseases of the respiratory and gastrointestinal tracts, and the use of *L. reuteri* NCIMB 30242 for the reduction of low-density lipoprotein cholesterol (reviewed in ref. 59). Although evidence exists for the use of VSL#3 in hepatic encephalopathy, evidence remains inadequate for its use in nonalcoholic fatty liver disease and alcoholic liver disease.[43,60] In reference to women's health, trials suggest benefits of *L. acidophilus*, *L. rhamnosus* GR-1, and *L. reuteri* RC-14 for vaginosis and vaginitis.[43,61–63]

11.3.2 Mechanisms of Action of Probiotics and Effects on the Gut Microbiome

The mechanisms of action responsible for the health benefits conferred by probiotics include direct or indirect impact on the gut microbiome and are either strain-specific or shared among probiotic taxa. Probiotics can directly impact the gut microbiome by: (1) immune stimulation for protection against specific microorganisms; (2) direct antagonism of microorganisms through the production of bacteriocins; (3) the production of substrates such as vitamins or exopolysaccharides required for the growth of other microorganisms; (4) fermenting and converting sugars into inhibitory products; and (5) competing for prebiotics for growth. Probiotics can indirectly impact the gut microbiome by: (1) competing for adherence to the epithelium; (2) enhancing intestinal barrier function; and (3) modifying intestinal properties for microbial colonization and persistence *via* reducing inflammation.[64]

Shared mechanisms of action among members of certain taxonomic ranks at the genus and species level have recently been recognized.[4] As reviewed in ref. 4, two genus-wide mechanisms exist for both *Lactobacillus* and

Bifidobacterium. One involves the presence of mucus-binding proteins that lead to the persistence of the probiotics in the gastrointestinal tract, and influence both competitive exclusion of pathogens and structural integrity of the mucosa, and immunomodulation.[4] The second involves the production of SCFAs, which play a role in intestinal motility, electrolyte transport, exert anti-inflammatory effects, and inhibit pathogens. At the species level, three mechanisms are shared amongst some species of *Lactobacillus* and *Bifidobacterium* including sortase-dependent pilin proteins with the same functions as mucus-binding proteins, bacterial unmethylated cytosine-guanine (CpG)-containing DNA which acts as a ligand for Toll-like receptor 9 (TLR9) whose signaling is important for intestinal epithelial homeostasis, and the production of high levels of vitamin B12 and folate by some *Lactobacillus* and *Bifidobacterium* species, respectively.[4]

Additionally, to provide an example of strain-specific effects of probiotic bacteria, the administration of three *Lactobacillus* strains, *L. acidophilus* Lafti-L10, *L. casei* CRL-431, and *L. rhamnosus* GG, to healthy adults revealed markedly different gene expression profiles as assessed from duodenal mucosa, with further analysis revealing that each strain induced differential gene-regulatory networks and pathways.[65]

While many mechanisms underlying probiotic action may target the microbiome, establishment of the probiotics in the intestinal tract is not necessary for them to exert benefits. To provide examples of the potential role of probiotics in modulating the gut microbiome, the literature was searched for studies reporting health benefits and analysis of gut microbiome in response to probiotics supplementation in healthy adults. The inclusion criteria for the studies outlined in Table 11.1 are as follows: (1) double-blind, randomized, placebo-controlled trials; (2) assessed probiotics as a singular dietary strategy; (3) assessed probiotics supplementation instead of probiotics in food; (4) reported significant results for probiotics supplementation compared to placebo (between-group analysis) instead of probiotics supplementation over time (within-group analysis); (5) reported health benefits; (6) reported analysis of gut microbiome; and (7) conducted on healthy adults.

A total of 10 human probiotic trials[66–75] were identified with objectives investigating the effect of probiotic supplementation on AAD,[66,72,73] appetite regulation and cardiovascular disease (CVD) risk markers,[67,68] impact on microbial ecology,[69] immune function and/or seasonal allergies,[70,74] insulin secretion and action,[71] and immune response.[75] The trials analyzed the gut microbiome using several different approaches and findings are summarized in Table 11.1. Four trials[67,68,71,74] reported health benefits that did not coincide with changes in the gut microbiome, suggesting that microbiome alteration is not necessarily associated with clinical outcomes; six trials[66,69,70,72,73,75] reported health benefits that coincided with microbiome changes, which depend on the probiotic that was studied and the conditions.

Table 11.1 Double-blind, randomized, placebo-controlled trials reporting health benefits and analysis of gut microbiome in response to probiotics supplementation in healthy adults.[a]

Probiotics, age[b,c,d,e] and sex[f], total daily dose, form[g,h]	Health benefits	Proposed mechanisms of action	Analysis of gut microbiome	Ref.
B. bifidum W23, *B. lactis* W18, *B. longum* W51, *E. faecium* W54, *L. acidophilus* W37 and W55, *L. paracasei* W72, *L. plantarum* W62, *L. rhamnosus* W71, *L. salivarius* W24, 25.5 ± 10.2 years[b,f], 1.0×10^{10} CFU[g]	↓ diarrhea-like defecations (frequency and consistency)	Impacting microbial composition and metabolic activity	↑ enterococci on D7 and D14	66
L. casei W8, 20–45 year[c,f], 10^{10} CFU[h]	↑ iAUC for fullness at W0 ↓ energy intake among women at W0 ↓ triacylglycerol at W4 ↓ C18 : 1n–9 among women at W4	Acute induction of appetite-regulating hormones and regulation of stearoyl-CoA desaturase-1 enzyme	↔ in α- and β-diversity	67, 68
L. paracasei DG, 34.9 ± 10.7 years[b,f], 2.4×10^{10} CFU[h]	*Overall* ↓ concentration of butyrate, acetate, and sum of 3 SCFAs *Subgroup* ↓ butyrate concentration in HB_{S10}	Impacting microbial ecology and changing/rebalancing SCFA concentrations	*Overall* ↔ α-diversity and number of genera Modified β-diversity (↑ absolute distances) ↑ *Proteobacteria* and *Coprococcus* and ↓ *Blautia* (↑ *Blautia:Coprococcus* ratio) *Subgroup* ↓ in sum of 6 genera of Clostridiales order: *Faecalibacterium*, *Blautia*,	69

	↑ butyrate concentration in LB_{S5}		*Anaerostipes*, *Pseudobutyrivibrio*, *Clostridium*, and *Butyrivibrio* in HB_{S10} ↓ *Ruminococcus* and ↑ unclassified Bacteroidales in LB_{S5}	
L. gasseri KS-13, *B. bifidum* G9-1, *B. longum* MM-2, 69.8 ± 0.7 years[d,f], 3.0×10^9 CFU[h]	↑ IL-10 in final period 1 and baseline period 2	Restoring gut microbiome and modulating immune response	↔ in α- and β-diversity ↑ Bifidobacteria and LAB and ↓ *E. coli*	70
L. reuteri SD5865, 49.0 ± 7.0 years[b,f], 2.0×10^{10} CFU[h]	↑ adaptation, disposition, and insulinogenic indexes	Incretin-mediated improvement in β-cell function	↔ in eubacteria, enterobacteria, and lactobacilli ↔ in relative abundance of bacterial taxa or OTUs ↔ in α- and β-diversity	71
L. rhamnosus R0011, *L. helveticus* R0052, 34.6 ± 1.2 years[d,f], 8.0×10^9 CFU[h]	↓ length of diarrhea-like defecations	Impacting specific microbial families involved in anti-inflammatory responses and inhibition of pathogen growth	↔ β-diversity ↑ *Porphyromonadaceae* and ↓ *Enterobacteriaceae* diversity	72, 73
L. gasseri KS-13, *B. bifidum* G9-1, *B. longum* MM-2, 26.0 ± 1.2 years[d,f], 3.0×10^9 CFU[h]	Improvements in MRQLQ global score and activity, nose symptom, practical problem, and other symptom domain scores ↓ constipation symptom scores at W3, W4, W6, and W7	Production of anti-inflammatory metabolites (SCFAs), secretory IgA production and reduced gut microbial translocation	↔ in α- and β-diversity	74

Table 11.1 (*Continued*)

Probiotics, age[b,c,d,e] and sex[f], total daily dose, form[g,h]	Health benefits	Proposed mechanisms of action	Analysis of gut microbiome	Ref.
L. johnsonii N6.2, 23.0 years[e,f], 5.0×10^{8} CFU[h]	↓ abdominal pain, indigestion, cephalic and gastrointestinal distress syndrome scores ↑ plasma tryptophan and ↓ kynurenine: tryptophan ratio of low to high LAB counts group after W12 Frequency alterations of immune cell subsets including ↑ activated Th1 and cytotoxic $CD8^{+}$ T cells after W8 and ↑ monocytes after W12	Impacting gut microbiome and altering immune responses by the metabolism of tryptophan	↔ in β-diversity ↑ *Christensenellaceae* after W12	75

[a]Abbreviations: B., *Bifidobacterium*; E., *Enterococcus*; L., *Lactobacillus*; CFU, colony-forming unit; D, day; iAUC, incremental area under the curve; W, week; SCFAs, short-chain fatty acids; HB_{S10}, 10 subjects with high butyrate concentration >100 mmol per kg of wet feces; LB_{S5}, 5 subjects with low butyrate concentration <25 mmol per kg of wet feces; IL-10, interleukin 10; LAB, lactic acid bacteria; *E. coli*, *Escherichia coli*; OTUs, operational taxonomic units; V, visit; MRQLQ, Mini Rhinoconjunctivitis Quality of Life Questionnaire; IgA, immunoglobulin A. Symbols: ↓, decrease; ↑, increase; ↔, no change; α, alpha; β, beta. Length of probiotics supplementation ranged from 2 to 8 weeks.
[b]Age: mean ± SD.
[c]Age range.
[d]Age: mean ± SEM.
[e]Median age.
[f]Males and females.
[g]Form: sachets with powder.
[h]Form: capsules.

11.4 Probiotics in Nutraceutical Supplements

Most of the probiotics available today comprise species of lactic acid bacteria (LAB, including *Lactobacillus*, *Lactococcus*, *Enterococcus*, and *Streptococcus*) and *Bifidobacterium*. *Bacillus* species are also used in addition to the strain *E. coli* Nissle and the yeast *Saccharomyces*. Probiotics are used either as a single strain or in a mixture of multiple selected strains.

11.4.1 Characterization and Taxonomic Identification of Probiotics

The characterization of new probiotic strains is performed as early as possible during the screening process. Culture-dependent and -independent techniques are applied to identify species and discriminate between closely related strains. *In vitro* testing assays combining biochemical (*i.e.*, nitrate reductase and catalase) and microbiological (viability and resistance to stress) methods are performed to phenotypically characterize the strain, while molecular techniques provide a deep identification and classification of the probiotic at the strain level. The FAO/WHO 2006 experts' recommendations suggested the use of DNA–DNA hybridization, DNA sequencing or other well-established molecular techniques for genotypic identification of probiotics.[76,77] PCR assays targeting specific genes or 16S and 23S ribosomal RNA (rRNA) offer a higher discriminatory potential,[78] and their use helps to not only identify probiotic strains,[79,80] but also to detect discrepancies in species designation and classification.[81,82]

Sequence-based identification and typing are cornerstone approaches for providing information on the degree of relatedness among various probiotic strains. In Figure 11.1, the 16S rRNA gene phylogenetic tree shows a clear separation between probiotic bacterial species and subspecies [Figure 11.1(A)], while the use of *hsp60* gene (coding for a 60 kDa heat shock protein) allows to distinguish between probiotic yeast and bacteria, and between different bacterial genera [Figure 11.1(B)].

The emerging use of comparative genomics has opened new challenging avenues for the development of probiogenomics to identify the core probiotic genomes and to predict the molecular basis for health-promoting activities of specific strains.[83]

11.4.2 Selection of Probiotics

Probiotic selection is an important and challenging process that requires knowledge about the physiology and genetics of candidate strains related to their behavior/role in the intestine, their functional activities, and interaction with other microorganisms (*i.e.*, pathogens). Selecting a probiotic candidate is driven by its potential to confer beneficial effects on the host and, according to FAO/WHO, must include safety, functionality, and technological aspects.[84] Most of the probiotics available on the market include

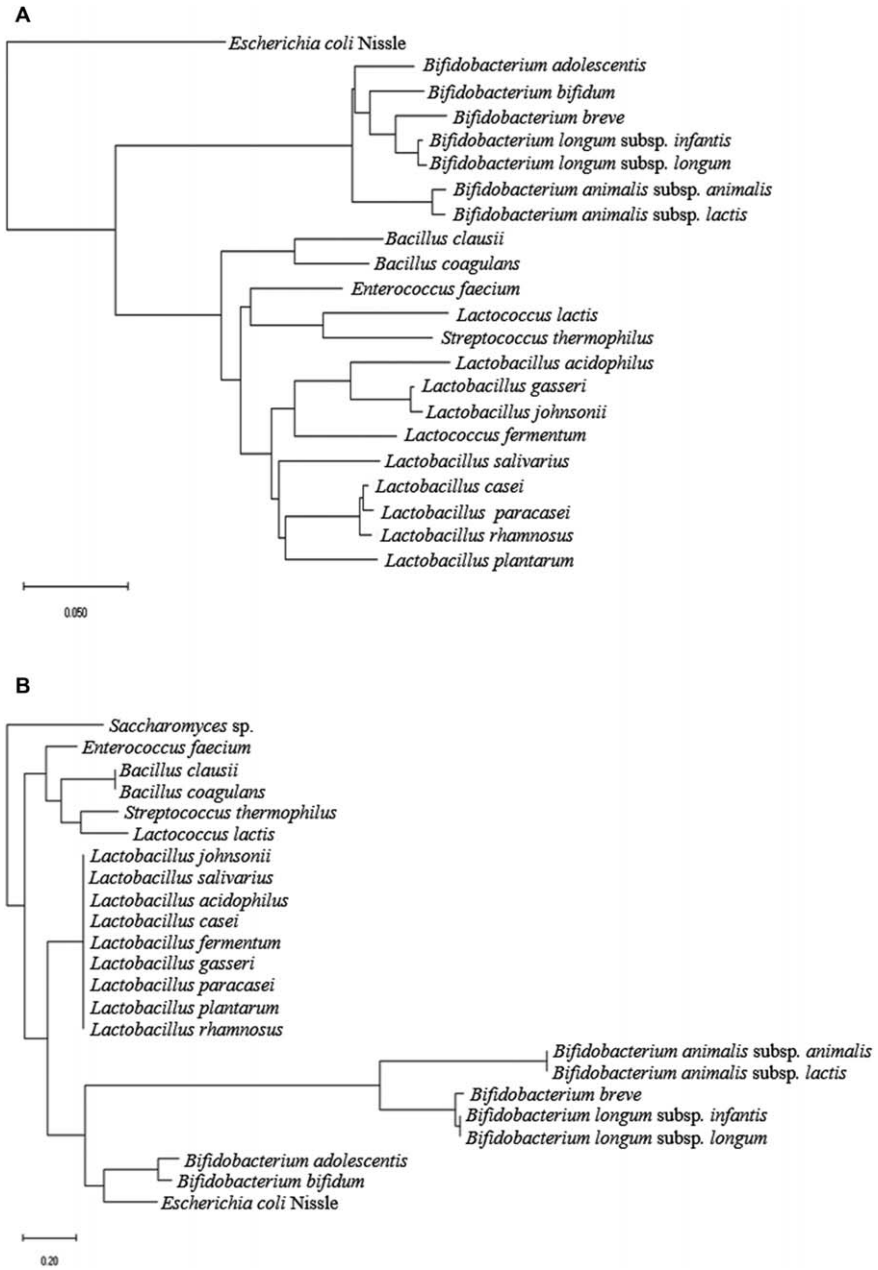

Figure 11.1 Dendrogram showing the phylogenetic relationship between probiotic bacterial species and subspecies (A) and between probiotic yeast and bacteria and between different bacterial genera (B) based on 16S rRNA gene and on *hsp60* gene sequences, respectively. The sequences were retrieved from GenBank and aligned using CLUSTALW algorithm and the MEGAX software. The phylogenetic trees were constructed from alignment results using the neighbor-joining algorithm based on the Jukes-Cantor model of nucleotide substitution (MEGAX). Bars (0.050–0.20) indicate nucleotide substitutions per site.

LAB with a long history of use as dairy starters or bacteria isolated from healthy humans.[85] These microorganisms are generally-regarded-as-safe, *i.e.*, non-pathogenic and not triggering allergic responses. For safety assessment for human use, the selection criteria must include records of isolation history, taxonomic identification, absence of virulence and transferable antibiotic resistance genes, ability to survive in the gastrointestinal tract, as well as assessment of side effects and effectiveness in human clinical trials (*i.e.*, Phase I and double-blind, randomized, placebo-controlled Phase II human trials or equivalent).[86] Furthermore, probiotics should be suitable for industrial processes, including cultivability on large scales and resistance to technological manipulations (*i.e.*, lyophilization), storage, and distribution.

11.5 Future Perspectives

Research on probiotics is continuously evolving and new concepts and new terms such as postbiotics and paraprobiotics are being introduced into the field. These terms have emerged as potential alternatives to the use of live microorganisms. Postbiotics, are non-viable bacterial products or metabolic byproducts derived from probiotic microorganisms that have biologic activity in the host,[87] and paraprobiotics, also called ''ghost probiotics'', refer to inactivated microbial cells or cell fractions which, when administered in sufficient amounts, confer benefits to consumers.[88] Other emerging terms include immunobiotics and psychobiotics referring to probiotics shown to be beneficial for immunity and mental health, respectively.[89] Moreover, probiotics are increasingly being studied in combination with prebiotics (synbiotics), leading to synergistic effects.[90] Lastly, there is certainly a greater need for research scientists and clinicians to design and conduct well-controlled human probiotic trials to substantiate probiotic efficacy with the ultimate aim of research translation into effective probiotic products for consumers in need. While probiotics are generally safe for use, their implementation in vulnerable populations should be supported by adequate trials for confirmed safety in practice.[91]

11.6 Conclusion

Undoubtedly, the dimensions of gut microbiome and probiotics research are expanding rapidly as more is learnt about their fundamental role in the promotion of health and treatment and prevention of chronic diseases. Probiotics confer numerous health benefits, their mechanisms of action can directly or indirectly impact the gut microbiome, and probiogenomics can be used as a new strategy to optimize selection of health-compatible microorganisms. While mechanisms and general benefits exist, which are shared among different probiotics, species- or strain-specific effects have been demonstrated and therefore substantiation should be sought for each probiotic product in the specific conditions and target population.

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CHAPTER 12

Clinically-relevant Herb–Drug Interactions: Current Status and Practical Considerations

BILL GURLEY

National Center for Natural Products Research, School of Pharmacy, University of Mississippi, Thad Cochran Research Center – Rm 2043, P.O. Box 1848, University, MS 38677-1848, USA
Email: bjgurley@olemiss.edu

12.1 Herb–Drug Interactions: A Brief Historical Perspective

For millennia, plants have been a source of food, drugs, and sometimes poisons. One could argue that humankind's ingestion of plants, more specifically phytochemicals in the form of plant secondary metabolites (PSMs), has held sway over the growth and advancement of our modern global society. As food sources, it is easy to grasp the impact that plants and their secondary metabolites have had on human civilization. Equally important, but more recent with regard to their cultural development and commercialization, is the role of PSMs as drugs. PSMs are not part of the primary biochemical pathways of plant cell growth and reproduction, but rather are involved in regulating symbiosis, defending against herbivores and pathogens, and inhibiting the growth of competing plant species.[1] As plants evolved and adapted to more difficult environmental stresses, so too did the variety, complexity, and toxicity of their resident PSMs. For centuries, various

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cultures have taken advantage of the pharmacological properties of PSMs present in either whole plants or plant extracts, properties that laid the foundational underpinnings of today's pharmaceutical industry.

To understand the important role plant extracts and PSMs have played in the evolution of modern medicine, one need only peruse an early volume of the United States Dispensatory (USD), a forerunner to the current United States Pharmacopeia.[2] For over a hundred years, American physicians and pharmacists relied upon the USD's scholarly monographs which detailed myriad formulations to be used in the treatment of various diseases; the vast majority of these monographs described plant-based preparations. Thus, it was clear that botanical extracts and their multitude of PSMs were a driving force in American medicine throughout the 19th and the first half of the 20th centuries. This ascendency of botanical preparations in the US and throughout the world, however, took a dramatic turn after World War II. It was at this time that the success of sulfa drugs and penicillins caused the American pharmaceutical industry to shift from multi-component, plant-derived preparations to purified, single-ingredient synthetic compounds. This reductionist approach to finding the purported pharmacological ''magic bullet'' for a particular disease all but ended the dispensing of and reliance upon botanical/herbal medicines. An eventual casualty in this reductionist trend was the teaching of pharmacognosy in America's colleges of pharmacy. Pharmacognosy—the study of drugs derived from natural sources, particularly plants—had been a mainstay of most colleges of pharmacy curricula for over a century, but by the 1970s this discipline had all but disappeared.

By the 1950s, conventional medications became firmly established as dosage forms containing synthetic, single-ingredient active pharmaceutical ingredients. As the number of new drugs increased, so too did the likelihood that patients might be prescribed multiple medications. Such practices inevitably gave rise to the risk and reality of clinically relevant drug–drug interactions. Drug–drug interactions are circumstances in which one or more drugs affect the efficacy or toxicity of another concomitantly administered drug. Drug–drug interactions may either be characterized as pharmacodynamic, where one drug elicits a pharmacological response similar or opposite to that of a concurrently administered drug; or pharmacokinetic, where one ''perpetrator'' drug adversely affects the metabolism and/or transport of a second ''victim'' drug, leading to markedly elevated or reduced blood/tissue concentrations of the ''victim'' drug that, in turn, leads to toxicity or reduced efficacy. Pharmacodynamic drug–drug interactions are usually the result of multiple drugs targeting the same receptor (*e.g.*, agonist *versus* antagonist, or substrates competing for the same receptor) or agents directed at different receptors whose activation give rise to a common biological response (*e.g.*, two or more antihypertensive medications working through different mechanisms). Pharmacokinetic drug–drug interactions are characterized by one or more drugs affecting the metabolism of another drug, often by inhibiting or inducing certain xenobiotic metabolizing

enzymes [*e.g.*, cytochrome P450s (CYPs), UDP-glucuronosyltransferases (UGTs), sulfatases (SULTs), *etc.*], or various transporters, be they efflux transporters [*e.g.*, ATP-binding cassette transporters (ABCs)], or uptake transporters [*e.g.*, solute carrier proteins (SLCs)]. (For overviews of the various drug metabolizing enzymes and transporters, their location in various organs and tissues, and how their modulation by phytoconstituents can affect the absorption, distribution, metabolism, and excretion of concomitantly administered drugs see ref. 3–6.) The descriptor, ''clinically relevant,'' refers to drug–drug interactions that result in marked pharmacological consequences that impact the health and safety of the patient.

Since the 1960s, the recognition, treatment, prediction, and prevention of drug–drug interactions has been a mainstay of the discipline of clinical pharmacology. In 1989, however, a new source of potential drug interactions came to light: phytochemicals. That year, the clinical significance of phytochemical-mediated changes in drug absorption was recognized when grapefruit juice (GFJ) was demonstrated to markedly enhance the oral bioavailability of several drugs with narrow therapeutic indices.[7] Furanocoumarins, a group of phytoconstituents present in GFJ, were found to inhibit intestinal CYP3A4, the most important human CYP with regard to the number (almost 50%) of prescription medications whose biotransformation is mediated by this enzyme. By inhibiting intestinal CYP3A4, GFJ furanocoumarins significantly reduced a major contributor to the ''first pass effect'' experienced by many orally administered drugs. By doing so, the toxicity of many drugs was dramatically enhanced when concomitantly ingested with GFJ. The GFJ issue, in turn, prompted a flurry of research into the effects of various fruit juices and fruit juice phytochemicals on human drug metabolism.[8,9]

Quickly on the heels of the ''fruit juice'' conundrum, the US Congress passed the Dietary Supplement Health and Education Act (DSHEA) in 1994.[10] With enactment of DSHEA, a profusion of diverse botanical products were released upon the US market. Coeval with the mass influx of botanical dietary supplements (BDS) onto US and global markets came apprehensions about the potential for BDS to interact with conventional medications. This concern was realized in 2000, when it was discovered that St. John's wort (*Hypericum perforatum*), a putative natural antidepressant, was convincingly linked to acute graft rejection in several solid organ transplant recipients who had ingested the popular BDS concurrently with the immunosuppressant cyclosporine.[11,12] Unlike the inhibitory effects of GFJ on CYP3A4, St. John's wort (SJW) was found to be a potent inducer of CYP3A4 expression. By inducing the expression of CYP3A4, as well as several other important CYPs and efflux transporters, SJW renders most drugs ineffective.[13] It was subsequently discovered that induction of CYP and ABC expression was attributable to hyperforin, a phytochemical unique to SJW and a potent ligand for the human pregnane xenobiotic receptor (hPXR).[14] It turns out that hyperforin is the most potent hPXR ligand ever discovered! As a key transcription factor regulating the expression of many xenobiotic metabolizing

enzymes and transporters, hyperforin-mediated activation of hPXR awakened researchers worldwide to the potential for BDS to trigger clinically relevant ''herb–drug'' interactions (HDI).

Drug interactions involving GFJ and SJW can be classified as pharmacokinetic HDI, in that phytochemicals (*i.e.*, furanocoumarins, hyperforins) were modulating the activity and/or expression of important xenobiotic enzymes and/or transporters, which, in turn, affected the pharmacokinetics and subsequent blood levels of concomitantly administered prescription medications. The second major class of HDI are those characterized as pharmacodynamic in mechanism.[15] Pharmacodynamic HDI are the result of phytochemicals exhibiting pharmacological actions that either bolster or negate those of conventional medications. In other words, pharmacodynamic HDI enhance or reverse the effects of traditional drugs without affecting their metabolism or transport. One of the best historical examples of a pharmacodynamic HDI involves *Ephedra*-containing BDS.[16,17]

Ephedra-containing BDS were sold in the US from 1994 to 2004, as diet aids, exercise performance enhancers, and ''energy boosters.'' From their inception, these multi-ingredient combinations of natural stimulants were linked to a host of cardiovascular and central nervous system adverse effects including hypertension, arrhythmias, heart attacks, strokes, seizures, and psychotic events. The number and severity of serious adverse health effects attributed to these controversial BDS eventually led to their removal from the market by the US Food and Drug Administration (FDA) in 2004.[18] At the heart of the controversy was the *Ephedra*-containing BDS formulation, which commonly contained extracts of *Ephedra*, natural caffeine sources (*e.g.*, green tea, guarana, kola nut, Yerba maté, *etc.*), as well as other natural stimulants (*e.g.*, yohimbe, citrus aurantium, cacao, *etc.*). The plant genus *Ephedra* is a source of ephedrine alkaloids (*e.g.*, ephedrine, pseudoephedrine, methylephedrine, norephedrine, norpseudoephedrine), which are recognized as indirect sympathomimetic agonists.[17] In fact, for many years, purified ephedrine, pseudoephedrine, and norephedrine were—and in some instances still are—sold as nonprescription bronchodilators, decongestants, and appetite suppressants, respectively. When combined with caffeine, the cardiovascular and central nervous system effects of ephedrine alkaloids are exacerbated to the point that over-the-counter (OTC) combinations of synthetic caffeine, ephedrine, and norephedrine (phenylpropanolamine) were removed from the US market in 1988 due to their untoward side-effect profile.[19] Unlike their synthetic OTC synthetic forerunners, *Ephedra*-containing BDS posed an even greater concern as many other naturally-occurring stimulants were incorporated into the formulation, most of which had excellent oral bioavailability. When taken concomitantly, these combinations of natural stimulants often intensified the effects of conventional prescription stimulants (*e.g.*, amphetamines, methylphenidate, phentermine, *etc.*).[17] On the other hand, concurrent ingestion of *Ephedra*-containing BDS with prescription antihypertensives (*e.g.*, diuretics, calcium channel blockers, beta-blockers, *etc.*), could abrogate blood pressure control.

Because many over-weight, hypertensive patients frequently consumed *Ephedra*-containing BDS as diet aids, such interactions were commonplace.[16,17] Thus, *Ephedra*-containing BDS provide an excellent historical case for clinically relevant pharmacodynamic HDIs.

12.2 Popularity of BDS and the Risks for HDI

For the last two decades, the global popularity of dietary supplements (DS) has been increasing. According to data from the 1999–2012 National Health and Nutrition Examination Survey (NHANES), 52% of the US population used dietary supplements, and among these 33% took multi-vitamin/multi-mineral products while 18% took BDS.[20] It appears that the majority of DS users only take one supplement but many take multiple products with approximately 10% ingesting more than four supplements concurrently.[20] Together, these data indicate that millions of Americans take dietary supplements and worldwide the numbers are likely to be far greater.

Current surveys indicate that 20–30% of prescription drug users take medications concomitantly with herbal supplements, oftentimes without notifying their health care professionals.[21,22] Moreover, 70% of regular users of BDS also take prescription medications.[21] Prospects for HDI, therefore, are considerable. Most vulnerable to these types of interactions are the elderly. Adults aged 65 years or older are the greatest consumers of prescription medications, and concurrent use of multiple medications (polypharmacy) is common in this population.[23] When coupled with age-related changes in drug disposition—a normal consequence of growing older—polypharmacy predisposes the aged to adverse drug reactions as well as drug–drug interactions. In the US, a noticeable increase in botanical supplement usage has occurred among seniors, with as many as 40–70% of survey respondents reporting regular use of herbal products.[22] Surveys also indicate that less than 50% of Americans over age 65 disclose their herbal supplement use to physicians.[24] This combination of polypharmacy, BDS usage, and reticence places the elderly at an increased risk of HDI.

12.3 Clinically Relevant Herb–Drug Interactions: Current Status

For almost two decades, a world-wide research effort has been underway to identify those phytochemicals and BDS that pose HDI risks.[3–6] Of the thousands of publications devoted to the topic, the vast majority describe *in vitro* or animal methods for estimating the likelihood that a particular phytoconstituent or BDS can produce clinically relevant HDI. Unfortunately, a number of shortcomings are inherent in these approaches that can reduce their predictive value. (For discussions of the precautions needed when interpreting the translatability of *in vitro, in silico*, and animal HDI studies please see ref. 3, 25–30). Clinical studies, therefore, are the gold standard for assessing the HDI potential of BDS.[30,31] Yet many clinical studies are also

subject to methodological flaws that can affect their quality.[31] Nevertheless, enough pre-clinical and clinical data has been generated to allow reliable predictions about the HDI risk for many popular BDS.

Tables 12.1 and 12.2 summarize the HDI risk for commonly used BDS obtained from original research and several exhaustive reviews published on the subject.[4–6,13,32–34] Surprisingly, despite the plethora of BDS on the global market, only a select few have been identified as noteworthy instigators of drug interactions. Briefly, those BDS posing the greatest risk for clinically-relevant pharmacokinetic-mediated HDI (Table 12.1) are St. John's wort (*Hypericum perforatum*), goldenseal (*Hydrastis canadensis*), black pepper (*Piper nigrum*), *Schisandra* spp., *Berberis* spp., and *Coptis* spp. These last five plant species all have secondary metabolites that share a common structural feature—methylenedioxyphenyl functional groups—the importance of which, from an HDI perspective, is discussed below. Three other BDS that pose an intermediate risk for pharmacokinetic HDI are green tea (*Camellia sinensis*), resveratrol, and quercetin. The reason these three BDS pose a lower risk has more to do with the limited number of quality studies verifying their clinical relevance rather than the therapeutic consequences of any resulting HDI. Table 12.2 presents those BDS recognized for their risk of producing clinically relevant pharmacodynamic HDI. Mechanisms underlying this category can be traced to the pharmacology of these BDS and whether they exacerbate or antagonize the pharmacodynamic effects of concurrently administered drugs.

12.3.1 Clinically Relevant Pharmacokinetic-mediated HDI (Table 12.1)

*12.3.1.1 St. John's Wort (*Hypericum perforatum*)*

More than 2000 peer-reviewed articles have been published on the safety and efficacy of SJW making it the second most studied botanical dietary supplement in the world, second only to caffeine-containing botanicals (*e.g.*, tea, coffee, others).[13,35] One reason for this scientific focus is SJW's recognized efficacy as a "natural antidepressant."[36] Many clinical trials have demonstrated that SJW's efficacy is both superior to placebo and comparable to several standard antidepressants, while engendering few side-effects.[37] This favorable risk : benefit ratio has made SJW one of the most readily consumed dietary supplements in the world. In turn, SJW's popularity has also exposed it as the most problematic BDS with regard to clinically relevant HDI.[13,35]

At the center of SJW's antidepressant activity as well as its HDI potential is hyperforin, a bicyclic polyprenylated acylphloroglucinol found exclusively in *Hypericum* species (Figure 12.1).[38] As an antidepressant, hyperforin functions as a broad-based neurotransmitter reuptake inhibitor, affecting the synaptosomal uptake of serotonin, dopamine, norepinephrine, glutamate, and gamma-aminobutyric acid with similar efficiency. The prenylated phloroglucinol is also a high-affinity ligand for human PXR, an orphan

Table 12.1 Botanicals associated with clinically relevant pharmacokinetic HDI.[b]

Botanical/ supplement	HDI risk	HDI mechanism	Phytochemical	Consequences	Precautions	References
St. John's wort (*Hypericum perforatum*)	High	Pharmacokinetic (induction of many CYPs and efflux transporters)	Hyperforin	Decreases Rx blood levels; renders most Rx ineffective (see Table 12.2)	Avoid concurrent use with all Rx	13, 35, 45–48
Goldenseal (*Hydrastis canadensis*)	High	Pharmacokinetic (inhibition of several CYPs and efflux transporters)	MDP-containing compounds: (berberine, hydrastine)	Increases Rx blood levels and risk of toxicity	Avoid concurrent use with all Rx	13, 109, 110
Black pepper (*Piper nigrum*)	High	Pharmacokinetic (inhibition of several CYPs and efflux transporters)	MDP-containing compounds: (piperine, piperamides)	Increases Rx blood levels and risk of toxicity	Avoid concurrent use with all Rx	13, 54, 111–115
Schisandra spp. (*S. chinensis; S. sphenanthera*)	High	Pharmacokinetic (inhibition of several CYPs and efflux transporters)	MDP-containing compounds: (gomisins, schizandrin, others)	Increases Rx blood levels and risk of toxicity	Avoid concurrent use with all Rx	13, 53, 116–118
Berberis spp. and *Coptis* spp.	High	Pharmacokinetic (inhibition of several CYPs and efflux transporters)	MDP-containing compounds: (berberine)	Increases Rx blood levels and risk of toxicity	Avoid concurrent use with all Rx	52, 119–121
Green tea (*Camellia sinensis*)	Intermediate to low[a]	Pharmacokinetic (inhibition of several uptake transporters; iron complexation in the gut)	Catechins: (EGCG, others)	May reduce absorption of select Rx, dietary iron, and folic acid	Avoid concurrent use with select Rx and MVMM	62, 65–69
Resveratrol	Intermediate to low[a]	Pharmacokinetic (inhibition of several CYPs and UGTs)	Resveratrol	May increase blood levels of select Rx	Avoid concurrent use with select Rx	71–74
Quercetin	Intermediate to low[a]	Pharmacokinetic (inhibition of CYP2C9, CYP2E1)	Quercetin	May increase blood levels of select Rx	Avoid concurrent use with select Rx	76, 77

[a]Risk based only on a small number of prospective clinical studies; need additional corroboration by other investigators before clinical relevance risk can be firmly established.
[b]CYP: cytochrome P450 enzyme; MVMM: multi-vitamin, multi-mineral supplement; spp.: species; Rx: prescription medication; UGT: UDP-glucuronosyl transferase; MDP: methylenedioxyphenyl.

Table 12.2 Botanicals associated with clinically relevant pharmacodynamic HDI.[c]

Botanical/ supplement	HDI risk	HDI mechanism	Phytochemical	Consequences	Precautions	References
Ephedra-containing DS	High[a]	Pharmacodynamic (cardiovascular and CNS stimulant effects)	Ephedrine alkaloids, caffeine, other natural stimulants	Increases BP, HR; increased risk of adverse health effects (*e.g.*, heart attack, stroke, seizure psychosis)	Avoid concurrent use with antihypertensive Rx, and Rx stimulants	16, 17
Ephedra-free DS	High	Pharmacodynamic (cardiovascular and CNS stimulant effects)	Synthetic+natural caffeine sources and other natural stimulants (*e.g.* yohimbine, synephrine, *etc.*)	Increases BP, HR; increases risk of adverse cardiovascular and CNS health effects	Avoid concurrent use with antihypertensive Rx, and Rx stimulants	17, 122
Liquorice root[b] (*Glycyrrhiza glabra*)	High	Pharmacodynamic (inhibits 11-β-hydroxysteroid dehydrogenase type 2)	Glycyrrhizic acid	Increases BP with prolonged use	Avoid concurrent use with antihypertensive Rx	86–88

[a]Due to increased risk of serious adverse health effects, *Ephedra*-containing DS were removed from the US market in 2004; some products are still available for purchase on the internet.
[b]This risk assessment does not apply to deglycyrrhizinated liquorice root products.
[c]BP: blood pressure; HR: heart rate; Rx: prescription medication.

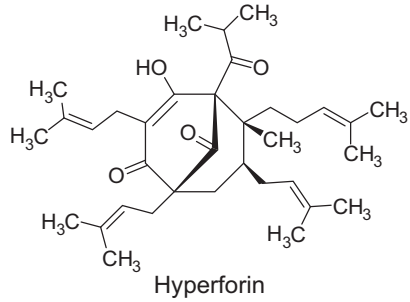

Figure 12.1 Chemical structure of hyperforin, present in St. John's wort.

nuclear receptor selectively expressed in the liver and intestine that mediates the induction of drug metabolizing enzyme and efflux transporter gene transcription.[14,39,40] Hyperforin's affinity for PXR (*i.e.*, $EC_{50} = 23$ nM) makes it the most potent activator of PXR ever discovered.[14] As a result, hyperforin induces the expression of the two most important CYP subfamilies involved in drug metabolism, CYP2C and CYP3A, as well as several ABC efflux transporters.[13,35] Accordingly, more than 70% of all prescription medications are susceptible to SJW-mediated interactions, the consequences being decreased oral bioavailability, enhanced systemic clearance, and reduced efficacy.

As with all botanicals, phytochemical content can vary considerably and such is the case with hyperforin.[41] Most SJW extracts are currently standardized to contain 3% hyperforin, yet many brands may possess amounts well below this value. Several clinical studies have demonstrated that SJW extracts containing less than 1% hyperforin are less likely to produce clinically relevant herb–drug interactions.[42–44] Unfortunately for consumers, few SJW products are specifically labeled as having low hyperforin content. Accordingly, to avoid significant drug interactions, consumers should avoid concomitant use of SJW and prescription medications. SJW's proclivity for producing HDI has been examined in many recent reviews,[13,35,45–48] the results of which are summarized in Table 12.3.

12.3.1.2 BDS Whose Phytochemcials Contain Methylenedioxyphenyl Moieties

The plant kingdom abounds with species possessing phytochemicals whose structures contain methylenedioxyphenyl (MDP) moieties (Figure 12.2). Popular BDS known to contain substantial quantities of MDP-containing plant secondary metabolites (MDP-PSMs) include goldenseal (*Hydrastis canadensis*), black pepper (*Piper nigrum*), *Schisandra* spp., *Berberis* spp., *Coptis* spp., and many others. Berberine and hydrastine are two isoquinoline alkaloid MDP-PSMs present in goldenseal.[13] Piperine and various other MDP-containing piperamides are present in *P. nigrum*; the gomisins,

Table 12.3 Drug interactions with St. John's wort confirmed in human studies.

Drug category	Effect of SJW on drug concentration
Antianginals	
Ivabradine	Decrease plasma level of drug
Antiarrhythmics	
Digoxin	Decrease plasma level of drug
Antibiotics	
Erythromycin	Decrease plasma level of drug
Anticoagulants	
Warfarin	Decrease plasma level of drug
Anticonvulsants	
Mephenytoin	Decrease plasma level of drug
Antidepressants	
Amitriptyline	Decrease plasma level of drug
Antifungals	
Voriconazole	Decrease plasma level of drug
Antihistamines	
Fexofenadine	Mixed, generally decreases plasma level of drug
Antihyperlipidemics	
Simvastatin	Decrease plasma level of drug
Atorvastatin	Decrease plasma level of drug
Anxiolytics	
Midazolam	Decrease plasma level of drug
Alprazolam	Decrease plasma level of drug
Quazepam	Decrease plasma level of drug
Antivirals	
Indinavir	Decrease plasma level of drug
Nevirapine	Decrease plasma level of drug
β-Adrenergic blockers	
Talinolol	Decrease plasma level of drug
Bronchodilators	
Theophylline	Decrease plasma level of drug
Calcium-channel blockers	
Verapamil	Decrease plasma level of drug
Nifedipine	Decrease plasma level of drug
Cancer chemotherapeutics	
Irinotecan	Decrease plasma level of drug
Imatinib	Decrease plasma level of drug
Corticosteroids	
Dexamethasone	Decrease plasma level of drug
Prednisone	Decrease plasma level of drug

Table 12.3 (*Continued*)

Drug category	Effect of SJW on drug concentration
Hormonal contraceptives	
Ethinylestradiol	Decrease plasma level of drug
Norethindrone	Decrease plasma level of drug
Ketodesogestrel	Decrease plasma level of drug
Hypoglycemic drugs	
Gliclazide	Decrease plasma level of drug
Tolbutamide	Decrease plasma level of drug
Immunosuppressants	
Cyclosporine	Decrease plasma level of drug
Tacrolimus	Decrease plasma level of drug
Nonsteroidal anti-inflammatory drugs (NSAIDs)	
Ibuprofen	Decrease plasma level of drug
Opioids	
Methadone	Decrease plasma level of drug
Oxycodone	
Proton pump inhibitors	
Omeprazole	Decrease plasma level of drug
Esomeprazole	Decrease plasma level of drug
Pantoprazole	Decrease plasma level of drug
Skeletal muscle relaxants	
Chlorzoxazone	Decrease plasma level of drug
5α-Reductase inhibitors	
Finasteride	Decrease plasma level of drug

schisantherin, schisandrin, among others, are common to *Schisandra* spp.; and berberine can be found in both *Berberis* and *Coptis* species.[13] In plants, MDP-PSMs often function as pesticides,[49] but when consumed by humans they can act as mechanism-based inhibitors of CYPs.[50] This type of enzyme inhibition is thought to arise from CYP-dependent oxidation of the methylenedioxy carbon to a carbene that subsequently interacts with CYP heme iron to produce a stable heme-adduct, termed a metabolic-intermediate (MI) complex.[50] It is through the formation of MI complexes that CYP isoforms are inactivated.[50,51] Because MDP-PSMs can function as mechanism-based CYP inhibitors, they present significant risks for HDI.

Several well-designed clinical studies have documented marked changes in the pharmacokinetic parameters of numerous drugs when taken in conjunction with MDP-containing BDS.[13] Most of these CYP-mediated changes can be ascribed to mechanism-based inhibition, whereas other alterations in drug bioavailability or clearance can be linked to inhibition of efflux transporter activity. In either case, the consequences can be especially

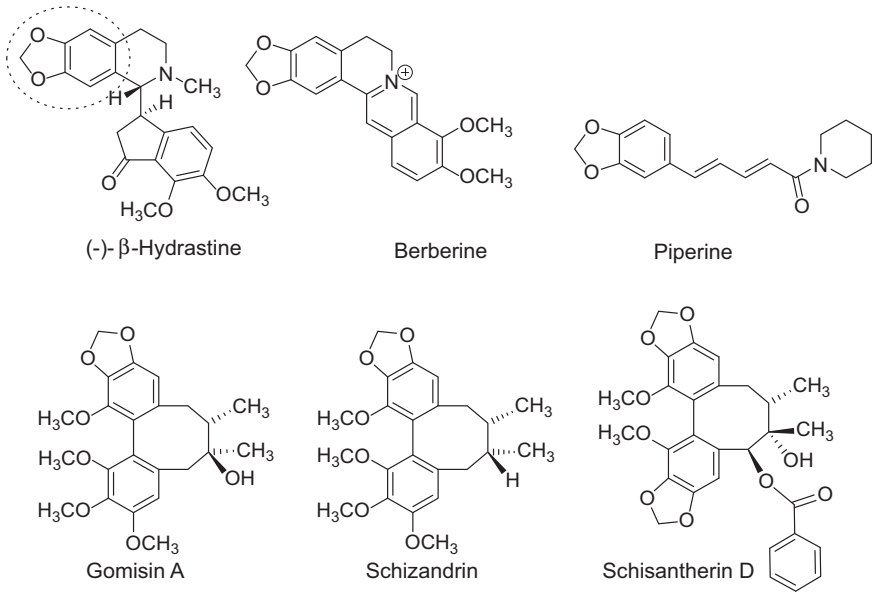

Figure 12.2 Representative methylenedioxyphenyl-containing phytochemicals (broken circle, methylenedioxyphenyl moiety).

problematic for drugs with narrow therapeutic indices like cyclosporine,[52] tacrolimus,[53] and carbamazepine.[54] This phenomenon is such that it has been taken advantage of by several supplement manufacturers who include black pepper extract or purified piperine into multi-ingredient BDS formulations as a means of improving the oral bioavailability of other phytochemicals.[55,56] Purified berberine is also gaining in popularity as an alternative medicine for treating diabetes and hypercholesterolemia,[57,58] while *Schisandra* extract, a popular traditional Chinese medicine, is commonly found in BDS targeted for weight-loss.[59,60] The presence of and the ability of MDP-PSMs to interact with conventional medications consumed by overweight or diabetic individuals often goes unrecognized, sometimes with dire clinical consequences, thus rendering their HDI risk high.

12.3.1.3 Green Tea Catechins

Green tea (*Camellia sinensis*) is one of the most popular and widely consumed beverages in the world. Numerous beneficial health effects have been attributed to a group of phytochemicals present in green tea, the catechins (*e.g.*, epicatechin, epicatechin gallate, epigallocatechin gallate, others) (Figure 12.3).[61] As a natural source of caffeine, green tea extract (GTE) is also a common constituent in many multi-ingredient, weight-loss BDS, and, when taken in excess, may give rise to pharmacodynamic HDI (see below). Human *in vivo* studies have demonstrated that green tea and GTE are not potent modulators of human CYPs;[62] however, recent clinical studies have

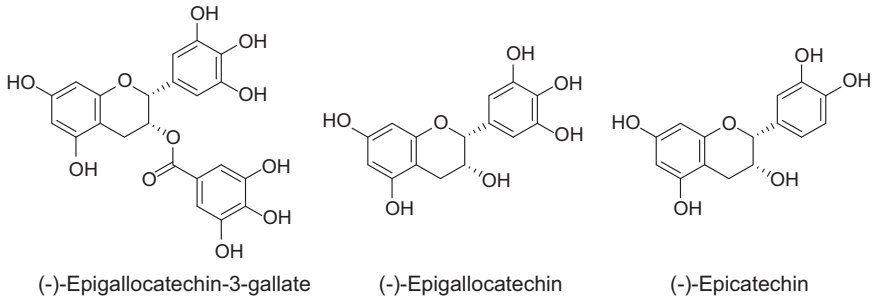

Figure 12.3 Chemical structures for representative green tea catechin polyphenols.

demonstrated that green tea is an inhibitor of several intestinal uptake transporters, namely organic anion transporting polypeptides (OATPs or SLCOs).[62–64] While the number of prescription medications that are exclusive substrates for these uptake transporters is limited, the inhibitory effects of green tea catechins on those drugs that are OATP substrates (*e.g.*, nadolol, digoxin) is clinically relevant.[65–67] In addition to their effects on OATPs, green tea catechins are also inhibitors of folic acid uptake and this mechanism is believed to be the result of catechin-mediated inhibition of the folate transporters SLC19A1 and SLC46A1.[68] Reduced folate exposure has been linked to fetal neural tube defects, and catechin-mediated reduction in folate uptake may be a significant herb–micronutrient interaction among pregnant women consuming green tea or green tea-containing BDS. Moreover, green tea catechins can also preclude dietary iron absorption by complexing ferrous ions in the intestinal lumen.[68] Many of the interactions related to green tea appear to be catechin dose-dependent,[8,69] although recent follow up data suggests otherwise.[66] Therefore, as a prudent precautionary measure, patients taking prescription medications or folate and/or iron supplements should limit their consumption of green tea beverages or green tea-containing BDS. In short, green tea's popularity combined with its inhibitory effects on various uptake transporters and dietary iron elevate the HDI risk for this BDS from low to intermediate.

12.3.1.4 Resveratrol

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is a natural phytochemical widely found in various plants (*e.g.*, *Polygonum cuspidatum*), fruits (*e.g.*, grapes, blueberries, raspberries, *etc.*), peanuts, and red wine.[70] It is a polyphenolic compound of low molecular weight that belongs to the stilbenoid family. Resveratrol naturally occurs in its glycosylated form, but its use as a BDS is primarily as the purified aglycone (Figure 12.4). It has been widely studied in clinical trials in the context of obesity, metabolic syndrome, heart disease, and cancer, among others; however, there are no specific recommendations concerning dosage and length of supplementation.[70] One reason for this uncertainty is resveratrol's poor oral bioavailability, which is why most

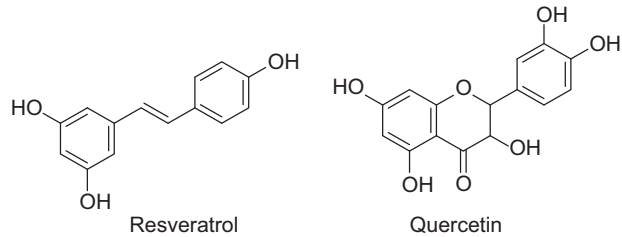

Figure 12.4 Chemical structures for resveratrol and quercetin.

commercially available products recommend daily doses of the purified aglycone in excess of 1 g. Owing to the popularity of resveratrol, its drug interaction potential has recently been evaluated in a small number of clinical HDI studies.[71–74] To date, resveratrol supplementation (500 mg once daily for 10 days, or 1 g once daily for 30 days) has been shown to inhibit the metabolism of substrates for CYP2C9 (*i.e.*, diclofenac, lorsartan),[71,73] CYP2D6 (*i.e.*, dextromethorphan),[71] CYP2E1 (*i.e.*, chlorzoxazone),[74] and CYP3A4 (*i.e.*, buspirone, carbamazepine).[71,72] Although the number of clinical investigations is limited, the results appear to be clinically significant. As a result, the current HDI risk for resveratrol can be classified as low to intermediate.

12.3.1.5 Quercetin

Quercetin (5,7,3′,4′-flavon-3-ol) is a polyphenol commonly found in many vegetables (*e.g.*, capers, onions, watercress), fruits (*e.g.*, elderberries, plums, apples) and other plants. Based on its prevalence in the food supply, quercetin is one of the most abundant dietary flavonoids. Like resveratrol, quercetin occurs naturally in its glycosylated form, its oral bioavailability is low and variable, and, as a BDS, is commonly sold as the aglycone (Figure 12.4). Based upon its *in vitro* antioxidant properties, quercetin has been touted as an adjunct treatment for various cancers and other chronic diseases, but its efficacy as a chemopreventative remains unproven.[75] Nevertheless, due to its acceptance and availability as a BDS, its HDI threat has recently come under scrutiny in two clinical studies. Quercetin supplementation (500 mg twice daily for 10 days) significantly altered the pharmacokinetics of diclofenac (CYP2C9 substrate),[76] and chlorzoxazone (CYP2E1 substrate).[77] These findings, while limited in number, augur a low to intermediate HDI risk for quercetin.

12.3.2 Clinically Relevant Pharmacodynamic-mediated HDI (Table 12.2)

12.3.2.1 Ephedra-*containing BDS*

With the passage of DSHEA in 1994, concentrated extracts of the plant genus *Ephedra*, a natural source of ephedrine alkaloids (*e.g.*, ephedrine,

pseudoephedrine, norephedrine, norpseudoephedrine, methylephedrine, and methylpseudoephedrine) appeared on the US and other global markets.[78] *Ephedra* extracts were frequently blended with extracts of natural caffeine sources (*e.g.*, guarana, green tea, kola nut, Yerba maté) and numerous other botanical extracts, many of which contained other sympathomimetic amines (*e.g.*, synephrine, phenylethylamine, octopamine) [Figure 12.5(A)].[78] Such complex botanical extract combinations were, and still are, known as "proprietary blends."[30] From 1995 until 2005, *Ephedra*-containing proprietary blends were marketed as thermogenic diet aids, energy boosters, and exercise performance enhancers.[17] Soon after their introduction, medical emergency departments and poison control centers began connecting serious adverse health events (*e.g.*, hypertension, heart attacks, strokes, seizures, psychosis, exertional heat illness) to *Ephedra*-containing BDS.[16,17] Subsequently, hundreds of adverse event reports were received by the FDA, and numerous case reports were published in the

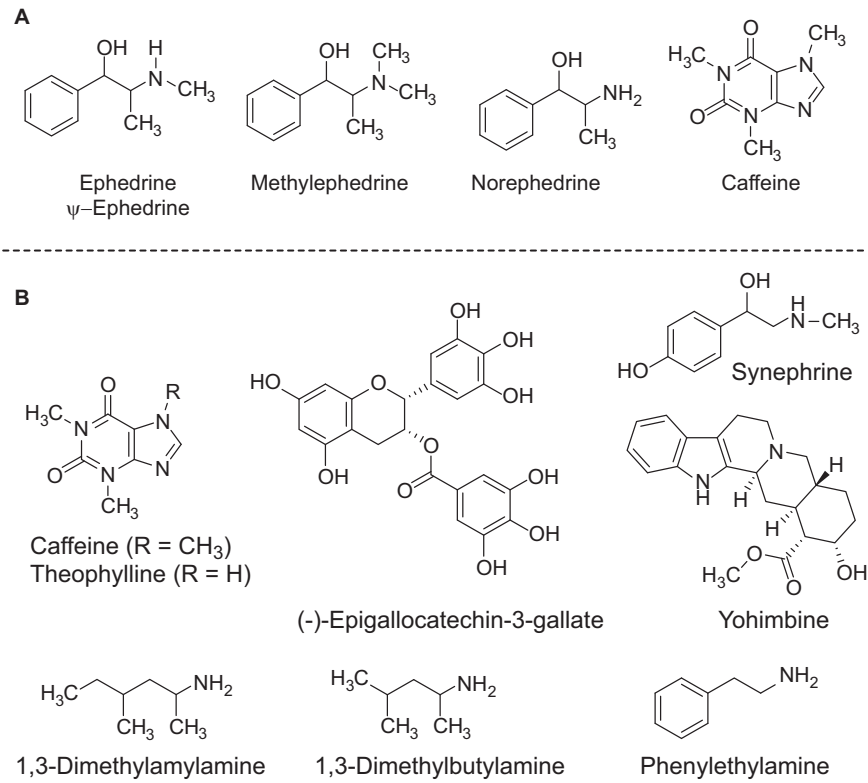

Figure 12.5 (A) Chemical structures for representative phytochemicals often present in *Ephedra*-containing BDS. (B) Chemical structures for representative phytochemicals often present in *Ephedra*-free BDS. (BDS containing 1,3-dimethylamylamine and 1,3-dimethylbutylamine are considered adulterated by the US Food and Drug Administration.).

medical literature substantiating the adverse effects of *Ephedra*-containing BDS. While the occasional case report or adverse event report may bring attention to potential safety concerns regarding BDS, they cannot establish causality; however, when thousands of adverse event and case reports continually link the same class of BDS to detrimental health effects, this evidence cannot be ignored. In 2001, *Ephedra*-containing products accounted for 64% of all BDS-related adverse events reported in the US while representing just 0.8% of all dietary supplement sales.[79] These adverse events were uncannily similar to those reported in the 1980s for nonprescription weight-loss products containing synthetic ephedrine, caffeine, and phenylpropanolamine; also known as ''amphetamine look-alikes''.[80] Health concerns prompted the FDA to ban ''amphetamine look-alikes'' in 1998,[19] but DSHEA made removal of *Ephedra*-containing supplements much more difficult.

The major concern with *Ephedra*-containing BDS was the unnatural combination(s) of multiple botanical extracts that would likely never be encountered as part of the normal diet. The reported cardiovascular and central nervous system (CNS) adverse events espoused an underlying sympathomimetic mechanism mediated by additive or synergistic pharmacodynamic effects of ephedrine, caffeine, and other phytochemicals (*e.g.* catechin polyphenols, yohimbine, forskolin, *etc.*).[16,81,82] It was also well known that caffeine could potentiate the sympathomimetic effects of adrenergic agents like phenylpropanolamine and ephedrine.[83,84] Moreover, caffeine and ephedrine, unlike most phytochemicals, are almost completely bioavailable with nearly identical time to peak plasma concentrations (T_{max}).[85] When administered as proprietary blends, the principal phytochemical offenders (*i.e.* ephedrine and caffeine) exhibited enhanced pharmacodynamic effects, making it difficult to differentiate their contributions from other phytochemicals present in the formula.[81,85] From an HDI perspective, *Ephedra*-containing BDS could impair the efficacy of conventional anti-hypertensive agents while intensifying the pharmacodynamic CNS effects of prescription stimulants like amphetamines or methylphenidate. As such, *Ephedra*-containing products were condemned for eliciting clinically relevant pharmacodynamic HDI. In 2004, the FDA removed *Ephedra*-containing BDS from the market, deeming them ''adulterated under the Federal Food, Drug, and Cosmetic Act because they present an unreasonable risk of illness or injury under the conditions of use recommended or suggested in labeling.''[18]

12.3.2.2 Ephedra-*free BDS*

With the banning of *Ephedra*-containing BDS, ''*Ephedra*-free'' substitutes—supplements formulated with higher quantities of caffeine (both purified and natural sources) but incorporating a greater selection of natural (*e.g.* phenylethylamines, synephrine, theophylline, yohimbine, *etc.*) (Figure 12.5B) and synthetic, albeit illegal, (*e.g.* dimethylamylamine, dimethylbutylamine, *etc.*) stimulants—instantly filled the void of their banned forerunners.[17] Today, a

wide-range of inadequately studied *Ephedra*-free BDS are marketed as weight-loss aids and exercise performance enhancers (*i.e.*, ''pre-workout'' supplements). Yet, despite their ''*Ephedra*-free'' connotation, these BDS do not appear to be any safer. With the introduction of *Ephedra*-free BDS in the mid-2000s coupled with the recent advent of caffeine-containing ''energy drinks/shots,'' reports of caffeine-related toxicity have steadily increased.[17] In addition, many consumers of *Ephedra*-free BDS often take prescription medications for underlying health conditions (*e.g.*, obesity, hypertension, attention deficit disorder). Accordingly, the effects of prescription stimulants (*e.g.*, amphetamine, diethylpropion, methylphenidate, *etc.*) may be amplified, while those of anti-hypertensive agents (*e.g.*, diuretics, calcium channel blockers, angiotensin receptor blockers, *etc.*) may be attenuated.[17] Therefore, even though these products are *Ephedra*-free, they are by no means trouble-free, as they carry a high risk for pharmacodynamic HDI.

*12.3.2.3 Liquorice Root Extract (*Glycyrrhiza glabra*)*

Liquorice has a very long history of medicinal usage in a number of cultures throughout the world.[86] Liquorice, specifically liquorice root and its extracts, has been used for a host of conditions and illnesses affecting the gastrointestinal, respiratory, cardiovascular, renal, neurological, and genitourinary systems.[86,87] Today, in many multi-ingredient weight-loss BDS, liquorice root extract is a common component. From a drug interaction perspective, liquorice may produce either pharmacokinetic or pharmacodynamic HDI;[86] however, the evidence for clinically relevant pharmacodynamic HDI is much stronger than the pharmacokinetic evidence.[87] The underlying mechanism for liquorice-mediated pharmacodynamic HDI can be traced to prolonged exposure to glycyrrhizin, one of the principal phytochemicals present in *G. glabra*, and its metabolites (*i.e.*, glycyrrhetinic acid) (Figure 12.6). Both glycyrrhizin and glycyrrhetinic acid can inhibit 11-β-hydroxysteroid dehydrogenase 2 (11βHSDH2), a key enzyme responsible for converting cortisol into cortisone. Inhibition of 11βHSDH2 leads to the accumulation of

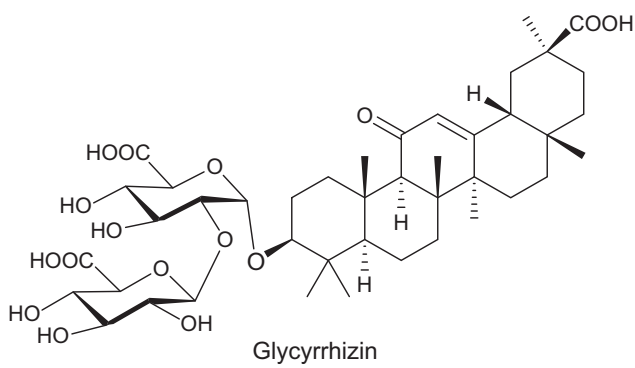

Figure 12.6 Chemical structure of glycyrrhizin, present in *G. glabra* extracts.

glucocorticoids with mineralocorticoid properties. In turn, the clinical manifestations of this inhibitory effect can be hypertension, hypokalemia, and cardiac rhythm disorders.[86–88] Drug classes that may be adversely affected by *G. glabra*-containing BDS include anti-hypertensives, antiarrhythmics, and other cardiovascular medications. It must be noted, however, that today many liquorice-containing BDS are deglycyrrhizinated and the risk for HDI with these preparations is markedly reduced.

12.3.2.4 BDS Usage with Warfarin

The anticoagulant warfarin is responsible for almost one-third of adverse drug events (*i.e.* hemorrhages) requiring hospitalization.[89] Warfarin's high adverse event rate is attributable to its narrow therapeutic range, which is influenced by a number of other factors including patient genetics, polypharmacy, age, and diet, to name a few.[89,90] Several common BDS (*e.g.*, *Ginkgo blioba*, garlic, ginseng, ginger) have often been cited as causative factors in HDI involving warfarin; however, the evidence for these interactions is often *via* case reports, *in vitro* experiments, or animal studies.[88] In contrast, prospective clinical studies assessing the pharmacodynamic and/or pharmacokinetic underpinnings for BDS-mediated interactions with warfarin have either been negative or inconclusive.[91–95] A good illustration of this *in vitro/in vitro* disconnect can be found with *Ginkgo biloba*. Several *in vitro* studies indicate that *G. biloba* inhibits platelet aggregation by interfering with platelet activating factor (PAF).[96] However, the phytochemical (*i.e.* ginkgolides) plasma concentrations necessary for inhibiting PAF are often orders of magnitude higher than those achieved *in vivo* in humans.[97] This lack of effect likely stems from significant inter-product variability in ginkgolide content and/or their meager oral bioavailability when administered as BDS.[98,99] It must be remembered that good phytochemical oral bioavailability is the exception in humans, not the rule. Nevertheless, given its capricious nature, a good practice is not to ingest any BDS with warfarin. In fact, it is prudent not to consume any BDS with any prescription medication that exhibits a narrow therapeutic window. For consumers unclear as to whether their medication(s) fall into this category, simply ask your pharmacist.

12.3.3 Why so Few Clinically Relevant HDI?

From the summaries above, readers may be surprised at the paucity of clinically relevant HDI candidates that have been identified so far. Popular, more recognizable BDS like black cohosh, curcumin, echinacea, garlic, ginseng, kava kava, milk thistle, saw palmetto, valerian, and many others either do not have phytoconstituents with potent pharmacodynamic effects or they are unable to modulate human drug metabolism and/or transport to any clinically relevant degree.[13] Such insufficiencies are often the result of physicochemical properties that preclude phytochemical absorption,

extensive pre-systemic metabolism, dosage formulation issues, or several other BDS-related idiosyncrasies discussed below.[3,29] Other factors that may come into play can be traced to methodological problems often associated with clinical HDI studies, several of which are also outlined below.

12.4 Key Factors When Conducting and Interpreting Clinical HDI Studies

12.4.1 Clinical HDI Study Designs and Outcomes

Clinical studies are the ''gold standard'' for accurately determining the drug interaction risk of a BDS.[30,31,100] As a general rule, approaches for conducting prescription drug–drug interaction studies are applicable to HDI studies with a few additional considerations. For a comprehensive discussion of the practical considerations involved in the proper design and conduct of clinical HDI studies see Gurley *et al.*[31] Perhaps the greatest obstacle to conducting a properly designed clinical HDI study is cost. Not all investigators have access to the proper clinical facilities and staff to conduct human HDI studies. This is why reports of HDI findings conducted *in vitro*, *in silico*, or in animals are more prevalent in the literature. Nevertheless, preclinical *in vitro*, *in silico*, and animal studies certainly play an important role in identifying those BDS that merit further clinical investigation, as it is simply implausible to prospectively screen all BDS for clinically relevant HDI. What follows, however, are a few key issues that must be taken into consideration when either conducting or evaluating a clinical HDI study.

12.4.2 Accounting for Phytochemical Content and Variability

Because BDS are composed of concentrated plant extracts containing a multitude of phytochemicals, their content can vary considerably. This variation is a result of differing species and cultivars, plant parts utilized, geographical location, time of harvest, extraction procedures, processing methods, and others, which not only affect botanical supplement quality, but also impact the outcome of clinical research into BDS.[101] One of the first, and perhaps most important, recommendations to consider when conducting HDI studies is proper phytochemical characterization of the BDS being studied. This is because product labels may not always accurately reflect the actual contents within the container. Moreover, label claims for individual quantities of phytochemicals are often incorrect. Significant variability in phytochemical content is not uncommon between brands of a particular BDS, but inconsistencies can also exist among different lots of the same brand.

Characterization comprises such aspects as identifying and authenticating both plant species and specific plant parts (*e.g.*, root *versus* leaves) utilized in producing a given extract. Equally important is identifying the extract type (aqueous/polar *versus* nonaqueous/nonpolar), ascertaining the phytochemical

profile, confirming individual phytochemical content per dosage form, gauging a dosage form's disintegration and/or dissolution profiles for marker phytochemicals, and finally, verifying that no adulterants and/or contaminants are present.[101] Due to the phytochemical complexity of BDS, special skill and instrumentation is needed to properly characterize a BDS. Ultra-high-performance liquid chromatography, gas chromatography, high performance thin-layer chromatography, centrifugal partition chromatography, mass spectrometry, nuclear magnetic resonance, and dissolution apparatus are just a few of the instruments needed to properly fingerprint and characterize a BDS prior to conducting pre-clinical or clinical HDI studies.

It is also important to realize that not all BDS are formulated to the same standards. Product label claims for botanical species and content may be inaccurate and there is little standardization within the industry. Conflicting published results on the HDI potential of a particular BDS are often due to one or more of the aforementioned issues. In some instances, the plant species/plant part and extract type can be verified and obtained from the formulation's manufacturer. Efforts to identify and authenticate a particular plant species within a BDS dosage form *via* DNA-based methods can be unsuccessful as the extraction process may leave behind little viable DNA.[102] In addition, many clinicians unacquainted with the ambiguities of BDS may not realize that different extraction processes can yield differing phytochemical profiles. For instance, an aqueous extraction method preferentially extracts hydrophilic compounds, whereas nonaqueous extractions remove more lipophilic components.

Verification of phytochemical content focuses on specific secondary metabolites recognized as ''marker compounds.'' Marker compounds are used to both identify a plant species and to standardize extracts prepared from that species. For example, ''marker compounds'' unique to milk thistle (*Silybum marianum*) include silybin A, silybin B, silychristin, isosilychristin, isosilybin A, isosilybin B, silydianin, and taxifolin, known collectively as ''silymarin.'' *S. marianum* extracts are typically standardized to contain not less than 90% and not more than 110% of the labeled amount of silymarin, consisting of 40–65% silybin A and B, 20–45% silydianin and silychristin, and 10–20% isosilybin A and B. Standardization requirements for marker phytochemicals present in most single-ingredient BDS marketed in countries across the globe can be found in their respective compendial publications (*e.g.*, Chinese Pharmacopoeia, European Pharmacopoeia, United States Pharmacopoeia, *etc.*). At the least, a comprehensive certificate of analysis should be requested from the BDS manufacturer specific to the lot number of the actual formulation proposed for study by researchers or clinicians. A better, albeit costlier, alternative is to have the BDS independently tested for phytochemical content by a laboratory with recognized expertise in botanical supplement analysis.[31] If an independent analysis does not produce a phytochemical profile characteristic for a specific plant species and extraction process, then the results of any HDI study conducted with that formulation are of limited use or altogether invalid.

12.4.3 BDS Dosage Form Performance

One explanation for why BDS clinical trials produce negative or contradictory findings can be traced to poor dosage form performance. Dosage form performance is defined as the rate and extent to which a tablet, capsule, or liquid gel capsule disintegrates and releases its active ingredients into gastrointestinal fluids under standardized conditions.[103] Phytochemical dissolution is reliant upon the molecule's physicochemical properties (*e.g.,* molecular weight, aqueous solubility, ionizability, *etc.*). Many phytochemicals are highly lipophilic and their dissolution into gastrointestinal fluids is significantly attenuated. Accordingly, inadequate phytochemical dissolution can negatively impact oral bioavailability.

From the perspective of dosage form performance alone, it comes as no surprise that many phytochemicals have poor oral bioavailability, given that the dissolution profiles for the vast majority of these marketed compounds are unknown. For example, dissolution standards have been established by the United States Pharmacopoeia for a very limited number of published botanical monographs (*i.e.*, curcumin, garlic, ginger, *Ginkgo biloba*, milk thistle).[104] BDS manufacturers are not mandated by current Good Manufacturing Practices (cGMPs) to perform dissolution or disintegration testing before product release; however, for those conducting clinical HDI studies this issue should be given serious consideration.

12.4.4 Novel DS Dosage Forms: Improving Efficacy or Augmenting HDI Potential

Recognizing that even conventional oral dosage formulations with favorable disintegration and dissolution characteristics may still result in poor absorption and bioavailability of many phytoconstituents, BDS manufacturers have begun to implement new extract formulation technologies to rectify this shortcoming. Some of the novel technologies currently under development are self-emulsifying microemulsions, nanoemulsions, nanoparticles, liposomes, Phytosomes®, incorporation of natural CYP3A4 inhibitors (*e.g.*, piperine, Schisandra, goldenseal), and others.[105] In each methodology, phytochemical dissolution and bioavailability can be demonstrably improved. Currently, only phytosomal preparations, a patented technology that complexes polyphenolic phytochemicals with phosphatidylcholine to form solid, liposome-like particles with extended shelf-life, are widely available. Milk thistle, green tea catechins, curcumin, grape seed proanthocyanidins, and *Ginkgo biloba* have been formulated as phytosomal preparations, and their polyphenolic marker compounds exhibit noticeably enhanced bioavailability when compared to conventional extract formulations. Time will tell whether these novel dosage forms portend a new era in BDS effectiveness, perhaps even tempering the medical community's skepticism about BDS efficacy. On the other hand, novel BDS dosage forms exhibiting substantially improved oral bioavailability, may bring about greater concerns

regarding toxicity and/or HDI—concerns heretofore overshadowed by inadequate dosage form design and performance. At present, very few of these new BDS dosage forms have been evaluated for their HDI potential.

12.4.5 Adulteration and Contamination Issues

Perhaps the most ignominious aspect of BDS affecting the entire industry is the prevalence of adulterants and/or contaminants among certain categories of BDS. The presence of heavy metals, microbial pathogens, pesticide residues, misidentified plant material, toxic plant extracts, active pharmaceutical ingredients, illicit anabolic steroids, and other unsuitable additives in many BDS has alarmed the medical community and lay public.[106] When conducting HDI research it is vital that the product under investigation be free of any adulterants and/or contaminants.

Despite inspections by regulatory agencies and implementation of cGMPs, the purposeful adulteration of BDS with conventional medications and illicit drugs still plague certain sectors of the industry.[106–108] Weight-loss, exercise performance enhancement, and sexual performance enhancement are the three main categories of BDS most often beset with adulterated products. It is not uncommon for certain weight-loss BDS to be spiked with anorexic agents (*e.g.*, lorcaserin, sibutramine, phentermine, *etc.*), while sexual performance enhancement products are frequently tainted with phosphodiesterase 5 inhibitors (*e.g.*, sildenafil, tadalafil, vardenafil) as well as unapproved analogs of these drugs. Exercise performance enhancers marketed for body builders are notorious for the addition of anabolic steroids, unapproved analogs of anabolic steroids, selective androgen receptor modulators (SARMs), and aromatase inhibitors (*e.g.*, exemestane). With respect to clinical HDI investigations, effects attributable to botanicals must be distinguished from those caused by purposeful and targeted adulterants.

12.5 The Current Knowledge Gap

Modern BDS have been on the global market for a quarter century, yet the lay public and medical community remain largely ignorant about the safety and efficacy of these products. Consumers often proclaim that ''supplements are natural and thus much safer than synthetic drugs,'' while health care professionals regularly assert that ''supplements are unsafe because they are unregulated.'' Such naive comments emphasize just how ineffective our educational efforts have been over the last two decades. In reality many, but far from all, natural products can be harmful when taken irresponsibly and in the wrong context, and BDS are indeed regulated, just not as rigorously as conventional drugs. In the 25 years since the passage of DSHEA, the knowledge base of most pharmacists and physicians regarding BDS is still regrettably lacking. In truth, most pharmacists at major chain drug stores have little input into the selection of those BDS which occupy significant shelf

space alongside nonprescription medications. They are, however, unfairly or not routinely consulted by patients and customers regarding many products and formulations. The pharmacist, therefore, is expected to serve as the on-site expert, and that is where the pedagogical need resides. Thankfully, for pharmacists and physicians at this day and time, the risk of clinically relevant HDI appears fairly low; however, the overwhelming majority of BDS remain to be evaluated, and so consumers and health care professionals alike should always err on the side of caution when it comes to ingesting BDS with prescription medications.

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