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Abstract: There has been a significant increase in the volume of research relating to antioxidants and health. The very nature of this research is inter-disciplinary, yet the full potential of such an approach, whereby nutritionists (clinicians), chemists, pharmacists and others all bring their expertise to bear in a concerted way, is rarely achieved. This is perhaps due to a lack of understanding of the methodology and terminology of the various disciplines. In this review, the terminology and features of nutritional studies are examined with particular emphasis on the confounders that may often be ignored by laboratory-based researchers. Attention is drawn to the potential role that ethics approval processes may have in directing outcomes.

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Abstract: There has been a significant increase in the volume of research relating to antioxidants and health. The very nature of this research is interdisciplinary, yet the full potential of such an approach, whereby nutritionists (clinicians), chemists, pharmacists and others all bring their expertise to bear in a concerted way, is rarely achieved. This is perhaps due to a lack of understanding of the methodology and terminology of the various disciplines. In this review, the terminology and features of nutritional studies are examined with particular emphasis on the confounders that may often be ignored by laboratory-based researchers. Attention is drawn to the potential role that ethics approval processes may have in directing outcomes.

Fereidoon Shahidi
Receiving Editor
Food Chemistry

Dear Fereidoon,

Thank you for reviewer's comments on our critical review titled "Nutritional Methodologies and their use in Inter-Disciplinary Antioxidant Research".

We have noted the reviewer's comments and made appropriate changes to address the various concerns and, in particular, to reduce the length of the manuscript. Thus, the text has been reduced from 31 to 25 pages and the Tables from 20 to 8 pages. The latter has been achieved by selection of representative studies to illustrate the diversity of research.

This paper was written specifically to address the readership of "Food Chemistry". As you will be aware antioxidant studies are extensive and every issue of the journal probably contains at least one paper addressing some aspect of antioxidant chemistry. This research is inherently interdisciplinary and yet the full potential of such an approach, whereby nutritionists (clinicians), chemists, pharmacists and others all bring their expertise to bear in a concerted way, is rarely achieved. We attribute this to a lack of understanding of the methodology and terminology of the various disciplines. This paper was written to address this need. Further reduction would compromise the message and yet submission to a different journal would also represent a compromise.

We hope that you are willing to consider this revised manuscript and that it meets with the approval of the reviewer.

Yours Sincerely,

Megan Kendall and Kevin Robards.

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Nutritional Methodologies and their use in Inter-Disciplinary Antioxidant Research

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Running title: Antioxidant nutritional methodologies

Abstract

There has been a significant increase in the volume of research relating to antioxidants and health. The very nature of this research is interdisciplinary, yet the full potential of such an approach, whereby nutritionists (clinicians), chemists, pharmacists and others all bring their expertise to bear in a concerted way, is rarely achieved. This is perhaps due to a lack of understanding of the methodology and terminology of the various disciplines. In this review, the terminology and features of nutritional studies are examined with particular emphasis on the confounders that may often be ignored by laboratory-based researchers. Attention is drawn to the potential role that ethics approval processes may have in directing outcomes.

Keywords: diet history, dietary trials, inter-disciplinary, nutrition.

Introduction

Population studies demonstrate that a lower incidence of cardiovascular diseases, some cancers, and other disease states associated with aging is experienced in subjects with diets in which fruit and vegetables predominate (Lock, Pomerleau, Causer, Altmann & Mckee, 2005). It remains unclear which specific 'nutrient' or mix of nutrients in fruit and vegetables is protective, or what the mechanism of action is. Nevertheless, antioxidants are viewed as primary contenders to explain the apparent protective role fruit and vegetables play in defending the body from the development of disease, and hence much research is being conducted in this area (Cooke, Evans, Mistry & Lunec, 2002; Dillard & German, 2000; Evans & Halliwell, 2001; Moller &

Loft, 2006). Plants are the ultimate source of these antioxidants and research on these nutrients has expanded greatly in the last decade as illustrated by the 71 and 946 papers published in 1996 and 2006, respectively[1].

Antioxidant research has involved *in vitro*, *ex vivo*, and *in vivo* studies, the latter involving both animal and human dietary trials. Much of this research, and particularly that performed by nutritionists, has involved the use of dietary supplements in human trials. The reported benefits of consuming these supplements are varied. A Danish study involving 43 healthy male and female non-smokers showed that consumption of supplements had a lesser effect on oxidative damage than fruit and vegetable consumption (Dragsted et al., 2004). While supplementation with some nutrients is medically sound, such as vitamin C treatment for scurvy, commercially available antioxidant supplements may be more commonly used by those wanting to increase their health and energy levels or as an attempt to prevent disease states. It appears that the majority of supplement users rate their health as good, are non-smokers, are physically active and consume at least 5 portions of fruits and vegetables per day (Hearnshaw, 2004). While antioxidant supplements are generally viewed as harmless (Lorenz et al., 2007) research into dietary supplements is increasing to determine the potential for both beneficial and harmful or pro-oxidant (an increased level of oxidative stress within the body) effects. With some antioxidant supplements there is a possibility of accelerating disease states in otherwise disease-free individuals (Angell & Kassirer, 1998; Heinonen et al., 1994; Omenn et al., 1996).

[1] ISI Web of Science search using the terms 'health' and 'antioxid*"

Although there has been an increased emphasis on antioxidants and health in the literature, no 'gold-standard' method exists for assessing the health effects of antioxidants, and as such many methodologies have been developed. Results are therefore difficult to compare, and because of this are often conflicting. While more research is needed to define the 'best' study design for antioxidant studies, collaboration between the different scientific disciplines undertaking this research is needed to limit gaps in the literature and to ensure efficiency. Although diverse groups have been involved in this research effort, generally they have worked in isolation. Thus, there is a need for understanding of the different approaches disciplines use. Of particular interest in this context are those tools, such as food diaries, commonly used by nutritionists which can be quite foreign to other disciplines. Protocols put forward by chemistry and pharmacy research groups would be of a higher efficacy if they employed nutrition monitoring and dietary collection methodologies. Trans-disciplinary research is the direction in which research is generally moving (Russel, 2000), and in antioxidant research it is to be greatly encouraged. Therefore, it is important that there is an understanding of the tools commonly used by nutritionists so correct implementation and interpretation of study results can occur. Even the use of human subjects, which is fundamental in many nutrition based studies, can be quite daunting to other discipline areas. While it is not a factor that must be controlled in laboratory work or animal studies, participant burden and hence compliance, is a serious consideration in human trials. If there is a high level of commitment required of the participants, the possibility of a high level of compliance is unlikely.

A dominant issue in performing human trials that is not immediately obvious to laboratory based researchers is the ethical questions that study designs may generate. What is considered 'ethical' in the field of nutrition research is modelled on the Declaration of Helsinki (Carlson, Boyd & Webb, 2004). The ethics approval process may impact what methodologies can be employed, and thus the completeness of the results and the level of understanding may be compromised.

This paper is limited to studies involving human trials and examines the strengths and limitations of the types of nutritional study designs and dietary collection methodologies employed in antioxidant research. This paper describes the common dietary collection methodologies, such as food records, used by nutrition researchers. This should allow those undertaking dietary antioxidant research, especially from a chemistry background, to more efficiently collaborate with nutrition researchers, and limit the gaps within the literature. This paper also comments on the role ethics can play in conducting human dietary trials.

Human Dietary Trials

In the broadest sense, human dietary trials can be divided under two distinct headings as observational and experimental based on whether or not there is an intervention (Peat, Mellis, Williams & Xuan, 2001). Observational, or cohort, studies do not involve any intervention or treatment. They are used to formulate hypotheses and to examine the relationships between dietary intake and disease development. They are also used to assess the incidence of disease, and the associations between exposure and subsequent development of a disease within a specific population group. The most important design feature is the population size. Experimental studies, on the

other hand, involve an intervention such as a change in diet in preventing or treating a disease state. While the population size may vary depending on the study design, subject selection is very important. Generally, the associations found in observational studies are tested in experimental studies.

Observational study designs

Cross-sectional, longitudinal and case-controlled studies as outlined in Table 1 are sub-divisions of observational studies. Cross-sectional studies (Coyne et al., 2005; Grievink, Smit, Van't Veer, Brunekreef & Kromhout, 1999; Strauss, 1999) are representative of a population at the specific point in time that information is collected. A major limitation of cross-sectional studies is they measure both outcome and exposure concurrently; no causal associations can be proven as it remains unclear if the disease state of interest, or exposure to a particular antioxidant, occurred first. This was the case in a study reporting a positive association between fruit and vegetable intake and pulmonary function (Tabak et al., 1999) in which a habitually low fruit and vegetable intake may have exerted a negative outcome on pulmonary function, or pulmonary function may already have been compromised regardless of the level of fruit and vegetable intake. While a relationship can be formed, a cause-and-effect conclusion can not be drawn. Another limitation with this design method is there is no consideration given to what has occurred in the study population before now, or what is to happen in the future, such as war, famine, or an infectious disease epidemic. The social environment may influence the development and prevalence of a disease state dramatically (Mackenbach & Howden-Chapman, 2003).

Longitudinal studies follow a population for a long period of time as in the case of a 24-year follow up to assess the association between dietary flavonoid intake and lung cancer risk (Knekt et al., 1997). Unlike cross-sectional studies, longitudinal studies document the incidence of disease development in a population, identify the risk factors of disease development including the political, social and environmental issues impacting on the study population and allow for a more holistic understanding of disease progression and prevalence (Geleijnse, Launer, Van Der Kuip, Hofman & Witteman, 2002; Hertog, Feskens, Hollman, Katan & Kromhout, 1994; Hertog, Sweetman, Fehily, Elwood & Kromhout, 1997; Peat et al., 2001).

In direct comparison to cross-sectional and longitudinal studies, case-controlled studies (Kelemen et al., 2006; Le Marchand, Murphy, Hankin, Wilkens & Kolonel, 2000; Shaheen et al., 2001) generally collect retrospective data. A case-controlled design involves recruiting those with a disease or characteristic of interest, and also enrolling those not experiencing the disease. Data representing past exposure is then documented and a comparison between the two groups can be made. For example, 607 adult asthma sufferers were matched with 864 controls to investigate for any protective effects flavonoids may exert on asthma severity (Shaheen et al., 2001). Retrospective data demonstrated that those flavonoids present in apples and red wine may have exerted a level of protection in those experiencing asthma. A nested case-control design refers to studies conducted within larger cohort studies. This method controls for any confounding time may exert as this sub-study occurs at the same time as the main study; confounders are factors associated with an outcome, but are not causal (Peat et al., 2001). For example, of those 22,000 healthy men who originally enrolled in the Physicians Health Study (PHS) (Gann et al., 1999), 578 developed

prostate cancer within the 13 years of follow-up. These men were matched to 1294 age and smoking status-matched controls, also from the PHS who did not develop prostate cancer during follow-up. The carotenoid lycopene was the only serum antioxidant found to be significantly lower in the cases as compared to the matched controls. 'Matching' of cases and controls allows for potential confounders, in this case smoking status and age, to be essentially eliminated, thus allowing for differences in exposure, such as lycopene intake, to be more accurately compared (Peat et al., 2001). This illustrates the need to select control subjects based on the identification of likely confounders in test subjects that must be matched.

Table 2 provides an overview of methodologies, durations, populations and results of representative observational, antioxidant studies demonstrating the diversity of the research that has been conducted in this area. For example, study durations ranged from 5 to 24 years (with the exception of cross-sectional studies where duration is not relevant) and involved diverse ethnic populations. The wide range in population size from hundreds to several thousands and outcomes is also notable. This diversity makes it difficult to compare results from the various studies. In some cases, the antioxidant investigations are secondary to the primary outcome of the study.

Experimental study designs

Blinding, randomisation (Crujeiras, Parra, Rodriguez, De Morentin & Martinez, 2006), utilising placebos (Blot et al., 1993) and employing cross-over designs (Beatty et al., 2000; Hercberg, Czernichow & Galan, 2006; O'reilly et al., 2001; Young et al., 1999) are all techniques used in experimental studies (see Table 3). Study techniques vary widely and they are not mutually exclusive. All techniques have inherent

limitations and strengths. Thus, a typical study design can incorporate numerous techniques to increase the validity of the results. For example, the Supplementation en Vitamines et Mineraux Antioxydants (SU.VI.MAX) (Hercberg et al., 1998a; Hercberg et al., 1998b; Hercberg et al., 1999), the Linxian China study (Blot et al., 1993), the α-Tocopherol β-Carotene Cancer Prevention study (ATBC) (Heinonen et al., 1994) and the β-Carotene and Retinol Efficacy Trial (CARET) (Omenn et al., 1996) were all randomised, double-blind, placebo-controlled primary prevention trials utilising large cohorts (Hemila, Virtamo, Albanes & Kaprio, 2004; Lee, Cook, Manson, Buring & Hennekens, 1999). Limitations such as compliance, age, and disease states can be controlled by employing different study techniques, as will be discussed in the next section.

Cross-over designs require subjects to participate in all the arms of the study. For example, in a randomised cross-over designed study assessing the effect dietary quercetin consumption exerts on preventing DNA damage in healthy subjects (Beatty et al., 2000) all subjects undertook a 14-day high quercetin diet and a 14-day low quercetin diet, incorporating a 14-day wash out period between the two interventions. The cross-over design is advantageous as any difference in outcome is measured in the same set of individuals, thus limiting inter-subject differences (Peat et al., 2001). 'Carry-over' is experienced when the effects of the first treatment impact the health of the subjects in such a way that it changes the outcome of the second treatment (Peat et al., 2001). As a fairly long wash-out period was implemented in this study, carry-over effects were limited. The authors gave no indication as to why a 14-day wash-out period was selected; however accuracy in selecting the duration of wash-out may have been strengthened had the authors delayed starting the next intervention until urine

and/or plasma levels of quercetin had fallen to baseline levels. Table 4 outlines the study areas, methodologies and populations used in various experimental studies within the scope of antioxidant interventions and associated health outcomes. Once again diversity is a feature of these studies as illustrated by the range of antioxidants studied and the varying number of participants. The latter ranged from 5 to approximately 40,000. Some studies were limited to male or female participants only and others were restricted to smokers or non-smokers. Study duration showed a wide range from weeks to years reflecting the nature of the study. Diverse outcomes have been reported. Supplementation significantly elevated plasma flavonoid levels in a study involving 18 female participants and rutin supplementation (Boyle et al., 2000). However, there was no change in oxidative stress as compared to placebo treatment. In contrast, olive oil consumption decreased oxidative stress and urinary 8-oxo-deoxyguanosine irrespective of phenolic content of the ingested oil (Machowetz et al., 2007). As with observational studies, the antioxidant investigations in some instances were secondary to the primary outcome of the study.

Randomised trials are used much more extensively than non-randomised (Kim, Kim & Sung, 2003). Based on results of various randomised clinical trials, Melton (Melton, 2006) concluded that the antioxidant concept was “a myth, a medical fairytale” despite the many observational/epidemiological studies that suggest a correlation of health and dietary phenol intake. This places randomised clinical trials in the *de facto* role of a gold standard. However, such trials suffer from time constraints relative to the lifetime development of a chronic disease. Is it surprising that we see contradictory data? Other factors that may limit or confound the results of dietary trials are elaborated below.

Limiting Factors in Human Dietary Trials

While many of the techniques outlined in Tables 1 and 3 can be used to increase the efficacy of study designs such as those summarised in Tables 2 and 4, there are factors that can limit outcomes of even the best designed studies which are outside the researchers' control. Such limitations, or confounders, include participant recall bias, participant burden, compliance of the subjects, duration of the study, demographics of the participants, known or unknown disease states of the participants, health behaviours of participants, and age and sex, and can compromise results (Peat et al., 2001). These factors need to be recorded during the study period to allow for control during the analysis and interpretation of results. While these aforementioned factors are due to the participant's levels of commitment, factors in which researchers can exert some control include the use of appropriate dietary measurement techniques and nutrient databases, as well as employing an attractive and ethically suitable experimental protocol to maximise subject recruitment and participation. By careful selection of the study protocol, less burden and hence more compliance from the participants should be experienced.

Age and sex of participants

Disease incidence increases with age. If subjects have sub-clinical development of diseases, such as impaired glucose tolerance or high cholesterol, results may be affected if these are not adequately accounted for during analysis. As the elderly have an increased risk of disease development, using an aged cohort is a means of 'selection bias' as only those who have survived to the inclusion age group, without major disease states, can participate (Hertog et al., 1994). It may be that these

individuals have led healthier lifestyles as younger people or have a favourable genetic make-up, reducing their risk for developing disease.

The CARDIA/YALTA (Hozawa et al., 2007) observational studies utilised a young American cohort, aged 18-30 years, in a bi-ethnic, multi-centre, prospective, longitudinal design. Serum carotenoid concentrations including α- and β-carotene, zeaxanthin, lutein and β-cryptoxanthin, and blood markers of inflammation, oxidative stress and endothelial dysfunction - all potential risk factors for disease development – were analysed. Serum carotenoids were found to be inversely related to markers of inflammation, oxidative stress and endothelial dysfunction. In a similar study design (Ito et al., 2005), utilising a cohort aged 39-79 years from a rural area of Japan, results indicated that high serum levels of carotenoids were also associated with a decreased risk of mortality from all causes of cancer. These studies suggest that age is not a confounding factor in carotene status and subsequent development of cardiovascular disease and cancer as both the young, and the middle aged-to-elderly cohorts experienced protective effects with higher serum antioxidant levels. However, it must be noted that by selecting an age group of 39-79 years, the authors have biased their results by selecting those who survived until this age inclusion criteria, and while there is no alternative it does eliminate a segment of the population without accounting for the reason of their absence (e.g. childhood disease). More proactive health behaviours over the life span may have played a role in these results. The associations with increased carotenoid levels should not override this.

Gender differences may also contribute to study results. Of particular interest are the differences in hormone levels especially in pre-menopausal women, and the use of

hormone replacement in post-menopausal women. Women also tend to have longer life expectancies, suggesting healthier habitual behaviours such as fruit and vegetable consumption, lower smoking levels and being less inclined to be overweight (Goldberg, Larson & Levy, 1996). These differences should be acknowledged when compiling study results of both male and female participants.

With large cohort studies, some interesting end points have been demonstrated when results are compared between men and women. In the SU.VI.MAX study (Hercberg et al., 1999) results after 7 ½ years of supplementation showed that total cancer incidence declined in the male participants (Galan et al., 2005; Hercberg et al., 2006). It has been hypothesised that this was due to the differences in baseline serum vitamin concentrations (Galan et al., 2005). The men who presented with low serum concentrations of antioxidant vitamins C and E as well as β-carotene experienced the greatest reduction in cancer incidence, compared to those with higher baseline levels. However, this same association was not found in the female cohort – there was no effect of supplementation seen on cancer incidence regardless of baseline serum concentrations. In fact, women typically presented with higher baseline levels of vitamin C and β-carotene, suggesting that the female participants may have habitually higher intakes of fruits and vegetables containing these nutrients. Other contributing factors may include the hormonal differences in the pre-menopausal subgroup, or lifestyle differences such as physical activity, smoking habits, alcohol consumption or a tendency to be less overweight (Galan et al., 2005; Hercberg et al., 2006).

While the SU.VI.MAX study (Hercberg et al., 1999) found differences between men and women, a comparison of the outcomes from the Women's Health Study (Lee et

al., 1999) and the Physicians Health Study (Cook, Lee, Manson, Buring & Hennekens, 2000) found no overall change in cancer incidence after β-carotene supplementation. Characteristics of the participants in the two studies were well matched. Each study protocol focused on health professionals, men aged over 40 years and women over 45 years, living in America, participating in the same supplementation protocol. Similar results irrespective of gender were also observed in the CARET study (Omenn et al., 1996). Within this study, both the men and women participating in the supplementation with β-carotene (30 mg/d) and retinol (25 000 IU) experienced an increased lung cancer risk by approximately 18%. Thus, when compiling results from a mixed cohort, care must be taken to ensure outcomes are independent of gender.

Baseline disease states/health behaviours

Health can be defined as the absence of disease. Recruiting a 'healthy' population is problematic as it is impossible to exclude those who are experiencing a disease state but are asymptomatic. While clinically diagnosed diseases are generally accounted for (i.e., cardiovascular disease, cancer, and diabetes), pathologies such as obesity, food allergies, and other risk factors of disease may go unaccounted unless a thorough physical examination is included in the study protocol. Health behaviours such as tobacco smoking, and drug and alcohol use also need to be considered when eliminating potential confounders of the results. As health behaviours tend to cluster, it is not unreasonable to suggest that subjects who are overweight, or engage in frequent tobacco or alcohol use, also participate in habitually less health promoting behaviours than subjects of normal weight and non-smokers/drinkers (Arts & Hollman, 2005; Serdula et al., 1996). These behaviours may contribute far and

beyond what effects an intervention in an experimental trial, or of a specific nutrient in an observational study, may exert over a specific health outcome.

In the Caerphilly study (Hertog et al., 1997), a male-only cohort was followed for 14 years to investigate flavonol intake and ischemic heart disease (IHD). Weakly positive associations were found, with the authors concluding that flavonols are not protective in reducing the development of IHD in this population. The baseline characteristics of the subjects suggest that tea accounted for 82% of flavonol intake. Those subjects with the highest tea intake were also more likely to smoke tobacco, and more likely to be manual workers, and thus exposed to air pollution (Caerphilly is classified as a light industrial town). Air pollution and tobacco smoke exposure are risk factors of cardiovascular disease development. The authors note that milk was normally added to the tea and this could have limited absorption of the flavonols due to chelation. While conflicting evidence of chelation effects has emerged (Lorenz et al., 2007; Reddy, Sagar, Sreeramulu, Venu & Raghunath, 2005), other interactions between molecules (Kang et al., 2004; Rohn, Rawel & Kroll, 2004) have not been investigated. Consideration of the baseline characteristics of subjects is important so correct conclusions are drawn. Risk factors of disease development, notably tobacco smoking, level of exposure to pollution, level of physical activity, and degree of obesity, need to be accounted for as these possibly confound the risk of disease development more so than the usual dietary intake of a potentially insignificant micronutrient.

Observational studies demonstrate that those with a high intake of fruits and vegetables containing β-carotene experience a reduced cancer incidence (Voutilainien

& Rissanen, 2004). The ATBC study (Heinonen et al., 1994) drew on this to implement a supplementation protocol including only males aged between 50-69 years who smoked at least five cigarettes per day. The ATBC study was a randomised, double-blind, placebo-controlled trial, and participants were supplemented with 50 mg/d vitamin E and 20 mg/d β-carotene. The authors' hypothesis was supplementation with these antioxidants may reduce the incidence of lung cancer in smokers. By eliminating all non-smokers, these results are more generalised to a smoking population, unlike other studies which do not solely include or exclude smokers (Arts & Hollman, 2005). It was found those undergoing β-carotene supplementation experienced a higher risk of lung cancer incidence, demonstrating that care needs to be taken when extrapolating findings to other population groups, and also for controlling, or eliminating confounding behaviours, as these may compromise results (Arts & Hollman, 2005).

Demographics

Where participants live determines what the 'normal' cultural diet, activity levels and health behaviours are, as well as social, environmental or political concerns. The results from multi-centre studies may be more generalised to the population at large than that of a specific population due to the diversity of the participants. However, extrapolating these outcomes to specific population groups may be quite inaccurate.

The population of Linxian County, China, experiences the highest prevalence of oesophageal and gastric cancers in the world (Blot et al., 1993). This population also demonstrates a low nutritional status. The Linxian study (Blot et al., 1993) was a randomised, placebo-controlled, double-blind design, with a supplementation

procedure consisting of combinations of retinol and zinc, riboflavin and niacin, ascorbic acid and molybdenum, and β-carotene, selenium and α-tocopherol. Supplementation lasted for 5 ¼ years. Those participating in the β-carotene, vitamin E and selenium arms of the trial experienced a significant reduction in cancer incidence, especially stomach cancer (reduction of ~21%). These results may be compared with the outcomes of the ATBC study (Heinonen et al., 1994) in which there was no decrease in lung cancer incidence with supplementation, and an increase in total mortality. However, the Linxian community experienced a stable level of subclinical deficiencies for several micronutrients whilst nutritional deficiencies were probably less prevalent in the Finnish population of the ATBC study. This level of nutrient deficiency may influence the interpretation of the two studies, as antioxidant supplement consumption in those with particularly low baseline levels of intake may experience beneficial health outcomes (as in the Linxian cohort) yet in well nourished cohorts antioxidant supplements may exert no effects or even pro-oxidant effects; this may be especially true if a pre-clinical disease state has initiated (Hercberg et al., 2006).

Duration of study protocols

The duration of a study protocol is very important. In trials assessing for the prevention of a disease the longer the study's duration, the more information is available and the more valid the results can become; this is true as long as the study participants have remained compliant. If sufficient time is not allowed for an effect to be seen or for correct associations to be made, the wrong conclusions may be drawn. In the Women's Health Study, supplementation with β-carotene lasted for ~2.1 years. In the Physician's Health Study supplementation with β-carotene lasted for ~12.9

years. While both studies followed similar supplementation protocols and neither found an overall effect of β-carotene supplementation on cancer development or mortality, comparison between the two studies may be inappropriate since the studies had such a difference in duration of supplementation. It must be remembered that disease development occurs over the lifespan. While five years is a long time for an experimental protocol to continue, in reality it only represents a fraction of a normal life expectancy. Therefore, documentation of the incidence of disease is limited in shorter trials. If, however, the duration of the study is too long and represents a high level of burden on the participants, compliance may decrease, confounding the results.

Presentation of study protocols

The presentation of the experimental protocol may have a dramatic effect in both the compliance of the participants and also the number of individuals willing to participate. Many factors may influence what is deemed appealing to different people, but factors such as a tablet/capsule versus a liquid or a food and beverage supplemented to the habitual diet, or to an experimental diet, may dictate the characteristics of the subjects who participate, and these characteristics may differ from those of the general population.

In a study involving cranberry juice consumption (Duthie et al., 2006), biomarkers pertaining to lipid and DNA oxidation did not change. Interestingly, no change in the level of catechins or anthocyanins was detected in the blood or urine after 2 weeks of cranberry juice intake. While this may indicate that these nutrients are not bioavailable from cranberry juice as noted by the authors, it may also be that the compliance of the subjects was questionable. No indication is given as to whether

compliance was monitored. It may be that the subjects did not adhere to the protocol because they simply did not like cranberry juice, and if this was the case no reliable information on bioavailability or biomarker status can be drawn. Also with a food or beverage protocol, increases in calorie intake may lead to changes in biomarkers that could impact results.

Techniques for measurement of dietary intake

Measurement of dietary intake is commonly used in nutritional studies (Beatty et al., 2000; Conquer, Maiani, Azzini, Raguzzini & Holub, 1998). The very act of measurement *per se* also has the potential to confound the study outcome. Dietary intake data are collected and analysed to both compare the average nutrient intakes of different participants or groups, and to determine a single participant's average intake. Results can be analysed to assess for change in habitual intake during a study period or to find associations between nutrient intake and disease incidence. Dietary intake data may be confounded by a number of factors such as habitual use of antioxidant supplements by subjects or a habitually high or low intake of foods such as fruit and vegetables containing the antioxidants of interest.

Quercetin supplementation using capsule intervention (Conquer et al., 1998) involved a lesser degree of burden by participants when compared to the ingestion of an onion cake and extra cup of tea (Beatty et al., 2000). As both studies monitored dietary intake during the study protocols, additional sources of quercetin, such as red wine, can be controlled for during the statistical analysis; the impact of quercetin from the intervention/supplement, and not that from the diet, can be assessed. The low-flavonoid protocol in the latter study (Beatty et al., 2000) required participants to

avoid flavonoid rich foods and beverages such as tea. Adherence to this requirement could have been difficult for some participants and unless problems with adherence were truthfully and adequately documented in the food records, would not be accounted for during analysis of the results. As a change in food intake may lead to less or more food being consumed, the nutrient status of the participants may have changed. Also if food intake did change, calorie intake could have increased or decreased, which may change the levels of oxidation markers in the blood and urine.

The three methods of dietary intake most commonly used in experimental and observational studies include food frequency questionnaires (FFQs), diet histories and food records. One draw-back of all of these techniques is that they rely heavily on the subject to be truthful; recall bias does occur which generally leads to under-estimation of energy and nutrient intakes (MacDiarmid & Blundell, 1997). For example, participants may be embarrassed about the quantity of food consumed (MacDiarmid & Blundell, 1997) or may selectively recall what they believe to be healthful and over-record their consumption of fruit and vegetables while under-recording un-healthful foods such as snack foods and alcohol (Rebro, Patterson, Kristal & Cheney, 1998). Depending on the burden of the study technique, participants may change their habitual diets, because it is easier to report/recall (MacDiarmid & Blundell, 1997; Rebro et al., 1998). Memory, especially for the elderly and the very young, can become a problem (Baxter et al., 2004; Vanstaveren, Degroot, Blauw & Vanderwielen, 1994) as is difficulty in estimating portion size (Blake, Guthrie & Smiciklaswright, 1989). A cross-checking procedure can be implemented (Calvert, Cade, Barrett & Woodhouse, 1997) in which two dietary collection methods, such as

a FFQ and food record are completed. These are then compared to give researchers an indication of any over- or under-reporting of intake.

The day-to-day variation of the diet is unaccounted with FFQs and short-term diet histories. Nevertheless, FFQs are often used as they are inexpensive to implement and are simple to administer and analyse (Lee & Nieman, 2003). FFQs consist of a list of foods and beverages and subjects are required to record how often a particular food is consumed with or without an indication of portion sizes (Kang et al., 2003). FFQs are affected by seasonal change (Fowke et al., 2004; Shahar et al., 2001) subjects may overestimate their yearly consumption of salad vegetables but underestimate their consumption of root vegetables if FFQs are documented in the summer months. An advantage of FFQs is they can be self-administered (Lee & Nieman, 2003) which limits the need to conduct interviews and hence decreases the time burden of both the participant and the researcher. Although FFQs employ a retrospective approach they are confounded by the participant's prospective, that is, their current, dietary habits (Fowke et al., 2004). Recently, a FFQ designed specifically for antioxidant studies has been generated; however more extensive validation is required before it can be utilised (Pellegrini et al., 2007).

A diet history is a more detailed clinical tool used by nutritionists. A diet history requires subjects to recall the types and amounts of foods and beverages consumed over a specific time period. As this is a retrospective procedure, recall bias can occur: many people may simply forget the types and quantity of food consumed, or inaccurately recall the consumption of snacks or beverages. As with FFQs, over- and under-reporting can occur. As dietary histories require an interview method, they can

become quite time consuming for the participant and researcher. Another limitation of diet histories is they do not allow for documentation of day-to-day variation within the diet; it is unusual for individuals to eat exactly the same foods in the same amounts each day (Bingham et al., 1994; Ma et al., 2006). A related approach is the 24-hour recall (Laurin, Masaki, Foley, White & Launer, 2004) as used for the estimation of flavonoid intake in the Australian population (Johannot & Somerset, 2006). Because the recall covers a very short time period that cannot reflect individual long-term intake, it is usual to collect data for several recalls. In one study, involving twelve 24-hour dietary recall interviews, about 80 percent of the total variance for each antioxidant could be attributed to day-to-day variation in individual intake (Hoffmann & Bergmann, 2004).

Food records are generally used in smaller studies, and their design allows for a more in depth analysis of food intakes compared to a FFQ; they give more data than a 24-hour recall or diet history, and account for the day-to-day variation of intake (Valtuena et al., 2007). Food records require subjects to document all food and beverages consumed on consecutive days within a specific time frame (usually 3-7 days) involving both weekday and weekend days as food consumption patterns change, especially during the weekend period (Haines, Hama, Guilkey & Popkin, 2003). Different approaches are used that vary in the level of burden for the participant (Kim et al., 1984). The complexity is further enhanced when foods are consumed in dishes that have many ingredients (e.g. spaghetti bolognaise) or when eating out at a restaurant. As may be expected, participants tend to simplify their diets for ease of documentation, which can lead to a reduction in food intake and variety of foods consumed (Rebro et al., 1998) and hence bias occurs.

Nutrient databases

Once food intake has been assessed using one or more of the above methodologies, it must be converted to a nutrient intake. This typically involves the use of nutrient databases that introduce a problem common to all dietary intake methodologies. Availability of analytical data is limited and databases are yet to be compiled that include all known foods and antioxidants. Tables which include these data are available in a variety of forms such as international, national, regional, food-industry and commercial databases. However, these databases are not entirely independent, since many of the basic data are shared. The need for an integrated approach to the development of food composition tables has been recognised (Burlingame, 2004) However, the preparation of comprehensive fully inclusive databases is a massive undertaking. If one considers that a typical food may contain between 100 to 1,000 compounds and this is multiplied by the number of foods, data collection becomes a daunting prospect. Ideally, food composition databases should provide average (or median) values for each nutrient, together with a statistical measure of variability.

The reliability of data in such databases depends on several factors. The compositional data must be truly representative of the food being consumed. Thus, the validity of nutrient databases is affected by seasonal variability, regional differences and species (Lee & Nieman, 2003). The degree of variation is typically much greater for micronutrients than for macronutrients such as protein and carbohydrate. For example, apple varieties can display a fifty-fold variation in total biophenol content (Manach, Scalbert, Morand, Remesy & Jimenez, 2004). Food composition data are typically collected over a long time period while at the same time food composition is

evolving due to changes in crop varieties and processing techniques. In the case of manufactured foods, formulations will also evolve to accord with latest dietary trends and health paradigms. Changes associated with stability and handling of a food introduce further variation. The moisture content of a food (e.g. 65-80% in cooked rice) will have an obvious impact on the reliability of data for other nutrients.

Data are often dependant on choice of analytical method and great skill and care are needed in the selection of an appropriate analytical method and in its application to ensure reliability of data (Hu & Liu, 2002; Smit, 2002). It is debatable as to what degree of change has occurred since 1943 when Widdowson and McCance (Widdowson & McCance, 1943) wrote: "There are two schools of thought about food tables. One tends to regard the figures in them as having the accuracy of atomic weight measurements; the other dismisses them as valueless on the grounds that a foodstuff may be so modified by the soil, the season, or its rate of growth that no figure can be a reliable guide to its composition. The truth, of course, lies somewhere between these two points of view." Therefore, our ability to interpret nutrient intakes is affected not only by the method of data collection, but is also affected by the availability and accuracy of nutrient databases.

Ethical considerations

Ethical standards help to ensure that consistent approaches and high standards are employed in studies involving human subjects. As has been reported in the cases of surgical (Panesar, Thakrar, Athanasiou & Sheikh, 2006) and medical research (Hearnshaw, 2004), the use of beneficial study designs and interventions in nutrition based research may also be compromised due to the level of ethical constraint

experienced by researchers. In a study comparing the ethical requirements between 11 European countries (Hearnshaw, 2004), all signatories of the Declaration of Helsinki, ethical approval processes were quite varied. While some countries did not require the approval of an ethics committee (Israel and the Netherlands), the length of time taken to gain ethical approval elsewhere (in particular the United Kingdom) took up to 10 weeks. This may therefore affect the amount of research conducted in certain, more ethically rigorous countries. While no specific example of ethics hampering nutrition research was found, the impact of ethics approval processes on implemented study protocols warrants an in-depth investigation.

Conclusions

While the study designs and dietary intake methodology of nutritional trials can be daunting, the utilisation of best analytical practice with nutritional methodologies allows for greater collaboration within the area of health and antioxidants, an essentially inter-disciplinary research topic involving interaction of nutritionists, (clinicians), chemists, pharmacists and others. Research in the area of dietary antioxidants is expanding, and is a subject that has many health and commercial applications. Rigorous study designs will assist in the discovery of the full benefits of antioxidants and their potential to prevent disease development.

Table 1: Study techniques used in observational studies and their features

Study technique	Important Features
Cross-sectional:	• examine a population's health and develop information regarding disease incidence and burden • assess relevant exposures or risk factors for disease development • rely on prospective data
Longitudinal:	• conducted over the lifespan/specific period • subjects are followed prospectively to assess health outcomes • these studies follow the progression of a disease state and hence identify risk factors and incident rates • large sample size and long follow-up required • generally more valid than cross-sectional or case-controlled studies in assessing risk factors of disease
Case-controlled:	• subjects with a known disease are matched with a control that does not have the disease • these studies rely on retrospective data • in a matched case-control study, cases are matched to the control by factors such as age, population and gender • characteristics/exposures are compared, and hence hypotheses of the cause of disease can be generated

Table 2: Observational Antioxidant Studies

Type of Study	Population	Antioxidant/ Daily Dose	Biomarkers	Duration	Methodology	Outcome	Results	Author
Cross-sectional population study	Data collected in the 1960s from 3325 adult males (Finland, Italy and the Netherlands)	N/A	N/A	Re-examined at 5 and 10 yrs	Diet history (6-12 months), pulmonary function by spirometric tests	FEV	Intake of fruit and vegetables positively associated with pulmonary function; level of antioxidant consumption (Vitamins C and E and β-carotene) not consistent with pulmonary function,	(Tabak et al., 1999)
Cross-sectional, population study	1651 subjects, aged ≥65y (Italy)	N/A	N/A	N/A	FFQ and neuropsychological dementia screening	Cognitive performance	A 'healthy diet' based on WHO recommendations, associated with better cognitive performance	(Leite, Nicolosi, Cristina, Hauser & Nappi, 2001)
Longitudinal, population-based study	9959 men and women aged 15-99yrs (Finland)	N/A	N/A	24yr follow-up	Health and demographic data, dietary history interview, 100-item FFQ	Total-cancer and lung cancer incidence	Inverse association between flavonoid intake and incidence of lung cancer	(Knekt et al., 1997)
Longitudinal, population based study	1239 males and 1943 females, 39-79yrs (Japan)	N/A	N/A	10.5 yr follow-up	Serum levels of carotenoids, retinol and tocopherol	Cancer at all sites, lung cancer, colorectal and	High serum levels of α- and β-carotenes and lycopene negatively associated with risk of cancers at all sites; β-cryptoxanthin showed a non-	(Ito et al., 2005)

						stomach cancer	significant inverse association with lung and stomach cancers	
Longitudinal, prospective, multicenter, epidemiologic study	5115 men and woman, aged 18-30yrs (America)	N/A	Plasma carotenoids, C-reactive protein, fibrinogen, superoxide dismutase, F2-isoprostanes, P-selectin, soluble ICAM1	15yrs follow-up	Demographics, anthropometry, smoking status, physical activity, diet history, blood collection at years 0, 5, 7 and 15	Changes in markers of inflammation, oxidative stress, and endothelial dysfunction and serum carotenoid levels	Higher serum carotenoid levels seem to exert a protective affect on markers for inflammation, oxidative stress and endothelial dysfunction	(Hozawa et al., 2007)
Nested case-controlled study (Zutphen Elderly Study)	470 men (Netherlands)	N/A	N/A	15y	Habitual dietary intakes, health history and activity levels assessed	Hypertension, CVD, death	Cocoa intake found to be inversely associated with blood pressure, CVD and all cause mortality	(Buijsse, Feskens, Kok & Kromhout, 2006)

FFQ- food frequency questionnaire; FEV- forced expiratory volume; CVD- cardiovascular disease; WHO- World Health Organisation.

Table 3: Study techniques used in experimental studies and their features

Study technique	Important Features
Randomised:	where neither the participant nor the researcher decides what treatment arm the participant is to enrol in; it is pre-determined
Non-randomised:	where the subject/researcher decides which treatment arm the participant enrols in
Single-blind:	the participant is not aware of what treatment they are participating in; the researcher does know
Double-blind:	where neither the participant nor the researcher is aware what treatment arm the subject is participating in at any stage of the trial
Placebo-controlled:	there is an inactive treatment; the subjects are unaware if they are enrolled in the active or inactive treatment
Cross-over design:	subjects are exposed to two or more treatments, with a wash-out period between each treatment; treatment effects can be assessed more accurately due to the limitation of inter-group confounding; 'carry-over' effects can affect the results

Table 4: Experimental Antioxidant Studies

Type of Study	Population	Antioxidant/Daily Dose	Biomarkers	Duration	Methodology	Outcome	Results	Author
Randomised, double-blind, placebo-controlled, primary prevention study	3146 men (45-60y) and women (35-60y) (France)	vitamin C: 120mg, vitamin E: 30 mg, β-carotene: 6 mg, Selenium: 100 μg, zinc: 20 mg, or placebo	serum levels of vitamin C, retinol, β-carotene, tocopherol, zinc and selenium	7.5 y	health history: smoking status, physical activity level, anthropometry, and 24-hr food records	FPG levels	antioxidant supplementation did not influence FPG after 7.5y; baseline dietary and plasma β-carotene concentrations inversely correlated with FPG	(Czernichow et al., 2006)
Randomised, double-blind, placebo controlled population-based study	38 445 female health professionals ≥45y; disease free (America)	100 mg aspirin every other day, and 600 IU of vitamin E every other day or placebo	N/A	mean follow-up of 6.9yrs	131-item validated SFFQ; flavonoid intake determined	reduction in cardiac or vascular disease/injury	flavonoid intake was not strongly associated with a reduction in CVD risk.	(Sesso, Gaziano, Liu & Buring, 2003)
Randomised, double-blind placebo-controlled,	578 males who developed prostate cancer, 1294	50 mg β-carotene, and aspirin every second day	N/A	13yrs follow-up	serum α-carotene, β-cryptoxanthin, lutein, lycopene, α-tocopherol, γ-	prostate cancer risk	lycopene exerted a significant inverse association on the development of prostate cancer; this relationship was confined to those men not assigned to take the β-carotene supplements	(Gann et al., 1999)

case-controlled study	matched controls aged 40-84yrs (America)				tocopherol, and retinol			
Randomised controlled study	283 high risk females (UK)	1000 mg/day vitamin C and 400 IU/day of vitamin E	N/A	second half of pregnancy	Doppler waveform analysis, blood samples, measurement of PAI-1 and PAI-2	PAI-1/PAI-2 ratio and frequency of pre-eclampsia	supplements had a significant reduction in pre-eclampsia in high risk females	(Chappell et al., 1999)
Double-blind, cross-over clinical study	27 men and women, aged 42 ± 2.6yrs (Canada)	250 mg/d quercetin supplements or placebo	N/A	28 d per protocol	anthropometry, 3-day food record, platelet aggregation, cholesterol and triglyceride measures, total phospholipids, fatty acid analysis, thromboxane production,	heart disease and thrombosis risk factors	supplementation markedly increased quercetin levels in the plasma; supplements failed to affect cardiovascular or thrombotic risk factors	(Conquer et al., 1998)

					plasma quercetin analysis			
Randomised, double-blind study	45 non-smoking males (Finland)	300 mg/d or 600 mg/d total phenolic compounds from oregano extract in orange juice or placebo	urinary excreted phenolic compounds, serum fatty acids, folate and homocysteine, TRAP, plasma F2-isoprotanes.	4 wk	4 d food records, limit consumption of high flavonoid containing foods/beverages, 24-hr pharmokinetic study	change in serum lipids and markers of antioxidant status and lipid peroxidation	no significant change in serum lipids or lipid peroxidation despite absorption (as determined by an increase is excretion of phenolic metabolites)	(Nurmi et al., 2006)
Randomised, placebo-controlled study	20 healthy females, 18-40yrs (United Kingdom)	750 ml/d cranberry juice or placebo	plasma total cholesterol, HDL-C, LDL-C, triglycerides; erythrocyes glutathione peroxidase, catalase, superoxide	2 wk supplementation, 4wks urine collection	blood pressure, anthropometry, diet questionnaire	change in plasma antioxidant activity, lipid peroxidation and DNA oxidation; bioavailability of cranberry anthocyanins	no plasma or urinary excretion of cranberry phenols detected; no change in lipid profile, cellular antioxidant enzyme activity, or DNA oxidation	(Duthie et al., 2006)

			dismutase; urine MDA, 8-oxo-dG					
Non-randomised clinical study	3 males and 2 females, aged 27±7.9, healthy (America?)	1200 mg Vitamin C, 1000 IU Vitamin E, 600 mg green tea extract, 530 mg grape seed extract, 600 mg olive fruit extract	8-isoprostanes	4hrs per supplemental protocol	Overnight fast, baseline and 4hr post-prandial plasma collection, FRAP	Change in 8-isoprostanes	Supplementation decreased 8-isoprostane formation	(Rabovsky, Cuomo & Eich 2006)

FPG- fasting plasma glucose; CVD- cardiovascular disease; 8oxo-dG- 8-oxo-deoxyguansine; MDA- malondialdehyde; FRAP- ferric reducing antioxidant power; HDL-C- high density lipoprotein cholesterol; LDL-C- low density lipoprotein cholesterol

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