Reliance on self-reports and estimated food composition data in nutrition research introduces significant bias that can only be addressed with biomarkers

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Abstract The chemical composition of foods is complex, variable, and depends on many factors. This has a major effect on nutrition research as it affects the ability to estimate actual intake of nutrients and other compounds, and the impact and consequences are largely unknown. Here, we investigate the impact of food content variability on nutrition research using three bioactives as model: flavan-3-ols, (–)-epicatechin, and nitrate. Our results show that the variability in the composition of the same foods impedes the accurate assessment of intake by the current approach of combining dietary with food composition data. This suggests that the results of many nutrition studies using food composition data are potentially unreliable and carry greater limitations than commonly appreciated, with considerable impact for dietary recommendations and public health. This limitation should be addressed by the development of better dietary assessment methods, in particular nutritional biomarkers.

Introduction

Nutrition is crucial for public health ***Global Burden of Disease 2017 Diet Collaborators*** (***2019***); ***The National Academies of Sciences and Engineering and Medicine Health*** (***2017***). However, despite considerable methodological progress, nutrition research still relies on self-reported dietary information and food composition data to investigate links between health and nutrition. Indeed, food composition data is the bedrock on which nutrition research rests today: it allows to estimate the intake of specific nutrients and other dietary compounds, and thus enables investigations into associations between nutrient intake and health outcomes. Such data inform policy makers in the development of dietary recommendations and risk assessments, and support the development of guidance for the general public and the food industry. However, this approach is not without significant challenges and limitations. One key challenge is the construction and maintenance of food composition data that underpin intake assessments for specific nutrients, as foods are highly complex and widely variable in their chemical makeup. Multiple factors affect the ultimate nutrient content of foods, including cultivar or breed, climate, growing- and harvest conditions, storage, processing, and methods of culinary preparation ***Greenfield and Southgate*** (***2003***). Even apples harvested at the same time from the self-same tree show more than a two-fold difference in the amount of many micronutrients ***Wilkinson and Perring*** (***1961***). Moreover, processed foods are

usually not standardised for composition but taste, texture and consumer preferences and thus vary in their chemical composition. Significant efforts have been made to generate extensive and detailed food composition tables, and complex sampling paradigms are used to obtain representative samples. Despite all these efforts, food composition data are generally used by relying on single point estimates, the mean food composition, de facto assuming that foods have a consistent composition. This approach introduces a considerable degree of error, bias, and uncertainty – and these are exacerbated by the limitations of self-reported dietary data which are known to carry substantial bias ***Subar et al.*** (***2015***).

Moreover, current approaches also assume that intake directly correlates with the systemic presence of a given nutrient, as it is through their systemic presence that many nutrients mediate much of their health-related biological effects. This introduces even more complexity when assessing true nutrient intake, as inter- and intra-individual aspects of absorption, metabolism, distribution and excretion, processes also impacted by the gut microbiome and other potentially highly variable and individual modulators of nutrient levels in the human body, should ideally be taken into account.

While all of this is well known in the nutrition expert community ***Gibney et al.*** (***2020***), the impact on both the interpretation of research findings and the development of dietary guidance and advice has been largely neglected, and there are only limited data exploring the impact on research outcomes ***Kipnis et al.*** (***2002***). It seems to be tenable that these limitations are a key contributor to the inconsistent and often contradictory outcomes of nutrition research and dietary guidance, which have received a high level of public attention and significant criticism in recent years ***Ioannidis*** (***2018***).

The EPIC Norfolk study (n=25,618, data available for 18,684 ***Day et al.*** (***1999***)) is ideally suited to investigate the impact of the variability in bioactive content on nutritional research because it has detailed dietary data based on the combination of self-reporting and food-composition data, nutritional biomarkers, as well as health endpoints, collected at the same time. Bioactives are food constituents that are not considered essential to human life but can affect health and are therefore extensively investigated. ***Ottaviani et al.*** (***2022***) We used three dietary bioactives as model compounds, including flavan-3-ols, (–)-epicatechin and nitrate (Table 1) as: i) there are widely-used food composition data tables used to estimate their dietary intake (Figure 1); ii) there exist suitable nutritional biomarkers, which can provide accurate information on actual intake ***Kaaks et al.*** (***1997***) and iii) there are data from dietary intervention studies that support associations between intake and health outcomes ***Larsen et al.*** (***2006***); ***Ottaviani et al.*** (***2018b***) (Table 1). For the purposes of this investigation, we determined bioactive intakes in a single cohort using data and samples collected at the same time. We used two different methods: the commonly deployed approached based on combing self-reported dietary intake with data from food composition tables (DD-FCT) as well as a method based on measuring nutritional biomarkers in urine samples (biomarker method). In the context of the first approach, we also considered taking into consideration nutrient content variability data provided by current food content tables. This was achieved by not only using single point estimates (mean values) as is common practice, but also by considering reported content ranges ***Blekkenhorst et al.*** (***2017***); ***Rothwell et al.*** (***2013***), using a probabilistic-type modelling approach. While our study focuses on bioactives, it is likely that the results will also apply to nutrients and other food constituents with high variability such as minerals, where more than two fold-variabilities were previously observed. ***Wilkinson and Perring*** (***1961***) and other nutrients including macronutrients such as fatty acids ***Reig et al.*** (***2013***); ***Schwendel et al.*** (***2015***). The findings of our study aim to test whether current approaches most often relying on the standardised, single point food content estimates obtained from food composition data can provide useful estimates of actual dietary intake and allow the investigation and meaningful interpretation of associations with health.

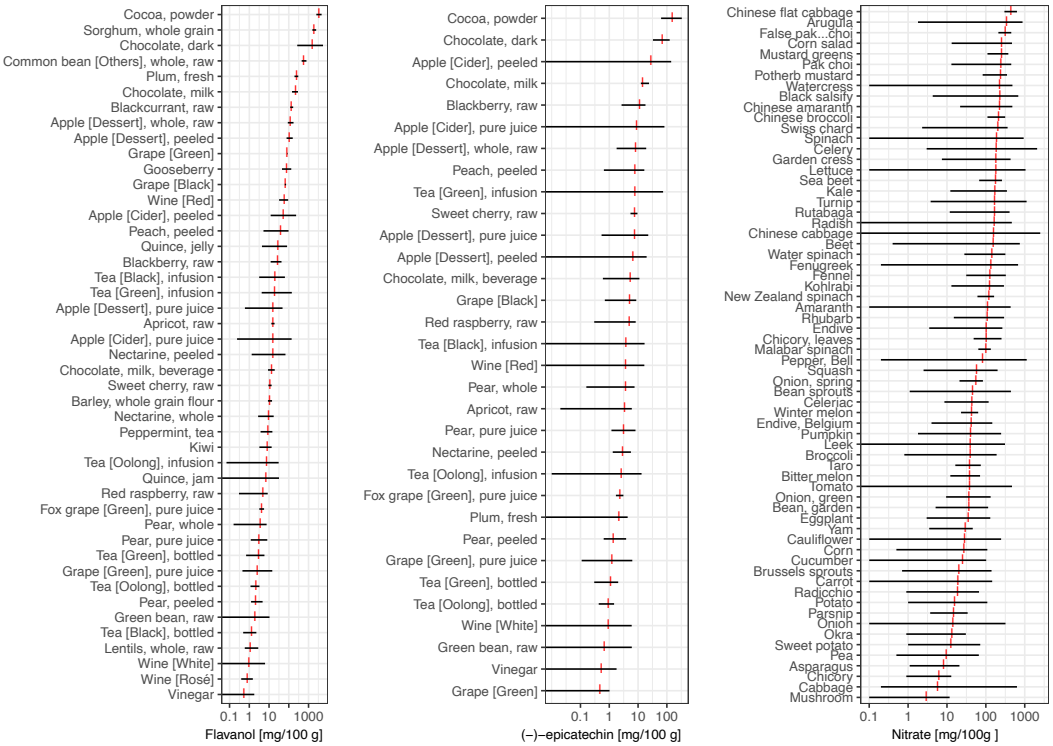

Figure 1. Variability in flavan-3-ol, (–)-epicatechin ***Rothwell et al.*** (***2013***) and nitrate ***Blekkenhorst et al.*** (***2017***) content of foods commonly eaten. Data show range of food content (black) and mean (red).

Table 1. Characteristics of dietary bioactives used as model system of dietary compounds to investigate the limitation of using single-point estimates of to assess intake and investigate health outcomes in nutrition research.

Dietary compound	Dietary distribution	Factors for variability	Biomarker of intake	Potential health effect
Flavan-3-ols	tea, apple and cocoa-derived products	cultivar, agricultural conditions, storage and processing	Urinary concentrations of gut microbiome-derived flavan-3-ol metabolites (phenyl-γ-valerolactone metabolites) ***Ottaviani et al.*** (***2018a***)	Reduce cardiovascular events and deaths ***Sesso et al.*** (***2022***). Reduce blood pressure ***Ottaviani et al.*** (***2018b***). Improve cognitive performance***Sloan et al.*** (***2021***)
(–)-epicatechin	tea, apple and cocoa-derived products	cultivar, agricultural conditions, storage and processing (including epimerization)	Urinary concentrations of structural related metabolites derived from phase II conjugation ***Ottaviani et al.*** (***2019***)	Improve vascular function ***Schroeter et al.*** (***2006***) ***Dicks et al.*** (***2022***) and reduce blood pressure ***Ottaviani et al.*** (***2018b***)
Nitrate	vegetables, drinking water	depends on a wide range of environmental factors such as fertilisation, light exposure and water supply	Urinary nitrate status can be used as a surrogate marker of intake ***Green et al.*** (***1981***); ***Pannala et al.*** (***2003***); ***Smallwood et al.*** (***2017***)	Dietary nitrate can reduce blood pressure ***Larsen et al.*** (***2006***)

Table 2. Study population and baseline-characteristics of 18,684 participants of EPIC Norfolk, for whom all data were available. Data shown are mean (SD) or absolute number and proportion. Data for urinary nitrate was available for 1027 samples.

	Women	Men
n	10167	8517
Age [years]	59 (9)	59 (9)
BMI [kg/m^2]	26.1 (4.2)	26.4 (3)
Systolic BP [mmHg]	134 (19)	138 (17.6)
Physical activity		
Inactive	2997 (30%)	2577 (30%)
Moderately Inactive	3258 (32%)	2096 (25%)
Moderately Active	2309 (23%)	1990 (23%)
Active	1603 (16%)	1854 (22%)
Smoking status		
Current	1121 (11%)	998 (12%)
Former	3250 (32%)	4647 (55%)
Never	5796 (57%)	2872 (34%)

Table 3. Intake of different bioactive compounds in EPIC Norfolk (median and IQR) when determined using different food composition data. Results are shown for estimates calculated using minimum, mean and maximum food content. and self-reported dietary data based on 24h diet recall (24HDR)

	Bioactive intake		
	Minimum food content	Mean food content	Maximum food content
Flavan-3-ols	48 (28 — 82)	120 (70 – 190)	329 (172 – 451)
(–)-epicatechin	1.5 (1.0 — 2.5)	19 (9 – 25)	33 (65 – 100)
Nitrate	5.5 (4.6 — 57)	100 (80 – 124)	204 (151 – 305)

Results

Impact of bioactive content variability when assessing dietary intake

The intake of an individual nutrient or bioactive is usually calculated by using self-reported dietary data and the mean food content as single point estimate. While the high variability in food composition is well known and recognised as a source of bias ***National Research Council . Coordinating Committee on Evaluation of Food Consumption Surveys. Subcommittee on Criteria for Dietary, Evaluation and ProQuest*** (***1986***), this is rarely acknowledged in such estimates and often assumed to have only little impact due to a regression to the mean. However, there is a paucity of data investigating the actual impact of this variability on estimated intakes. We have estimated the potential impact of the variability in flavan-3-ols, (–)-epicatechin, and nitrate food content on estimated intakes of the respective compounds and compound classes in 18,684 participants of EPIC-Norfolk for whom all relevant data were available (Table 2). Table 3 shows a comparison of estimated intakes when calculated using the DD-FCT approach with mean food content, as is current practice, as well as minimum and maximum reported food content. These results demonstrate a large uncertainty for estimating actual intake when taking the large variability in bioactive content into consideration. In comparison to the uncertainty introduced by the variability in food composition, the uncertainty associated with the use of self-reported methods of 2% to 25% ***Stubbs et al.*** (***2014***) appears to be small. There is an overlap in the possible range of bioactive intake between study

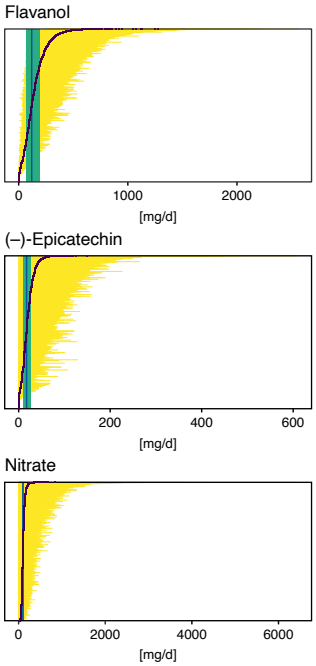

Figure 2. Possible intake ranges of flavan-3-ols, (–)-epicatechin and nitrate in each individual study participant displayed from low to high possible bioactive intake level. Range of bioactive intake was calculated using an approach similar to probabilistic modelling by sampling randomly from the distribution of possible food composition (n=10,000 iterations). Intake based on mean bioactive content, as is common practice, is indicated by a red line. Green line shows median intake of the entire cohort and the green box the inter-quartile range.

participants (Figure 2), making it difficult to identify low and high consumers or to rank participants by intake (see also below). These results show that bioactive content variability significantly contributes to the uncertainty in the estimation of dietary intake, even more than the error incurred by self-report methods that have attracted a lot of attention and discussion in nutritional research ***Subar et al.*** (***2015***).

Impact of food composition variability when assessing relative intake

In many studies relative instead of absolute intakes, for example quintiles, are used ***Altman and Bland*** (***1994***). It is assumed that relative intake is less affected by measurement error than absolute intake, and thus can mitigate some of the limitations of estimating dietary intake ***Streppel et al.*** (***2013***). We have therefore investigated how the ranking of participants is affected by the variability in bioactive content and compared the relative intake of participants with low (p25 – based on mean bioactive content), medium (p50) and high (p75) intake. Bioactive content variability was introduced in the analysis using an approach similar to probabilistic modelling by sampling randomly from the distribution of possible food composition for each food consumed by each participant. Figure 3 shows the result of 10,000 of such simulations. They suggest that the high variability in bioactive content makes estimates of relative intakes unreliable. Indeed, depending on the actual food consumed, the self-same diet could put the self-same study participant in the bottom or top quintile of intake. This suggest that it is difficult to obtain reliable relative intakes from dietary data alone, and that ranking by those data is unreliable.

In order to confirm the findings of our simulations, we have compared relative intakes estimated using data from DR-FCT and biomarker method. The biomarkers used in this study ***Green et al.*** (***1981***); ***Ottaviani et al.*** (***2018a***, 2019); ***Pannala et al.*** (***2003***); ***Smallwood et al.*** (***2017***) have been validated and characterised previously (Table 1) and are suitable to estimate relative intake***Keogh***

et al. (***2013***). Like the 24h dietary recall data used here, they reflect acute intake. Intake estimated from DR-FCT method was calculated using the common approach based on mean bioactive content in databases. The association between this self-reported intake and biomarker is weak with a maximum Kendall's τ of 0.16 (for (–)-epicatechin) and lower for flavan-3-ols (0.06) and nitrate (-0.05). Figure 4 illustrates this by comparing respective quantiles of intake, as these are commonly used to categorise relative intake. The data show very modest agreement between the two measurement methods (only 20% to 30% of participants assigned to the same quantile) and confirm that ranking is not suitable to address the measurement error and uncertainty introduced by the high variability in bioactive content. Overall, this shows that relying on a single value of bioactive content in food for all participants introduces bias when assessing relative intake of dietary compounds.

Impact of bioactive content variability on estimated association between intake and health endpoints

We have shown above that the high variability in food composition has an impact on estimates of intake using the DR-FCT method. However, it is not known whether this affects estimated associations between intake and health endpoints. Here, we use simulations to explore how the variability in food compositions affects such estimates in a "vibration of effects" type approach ***Patel et al.*** (***2015***), and compare these with results derived from biomarker estimated intakes. We use the cross-sectional association with blood pressure as example, as all three compounds have a well-established acute effect on vascular function ***Larsen et al.*** (***2006***); ***Ottaviani et al.*** (***2018b***); **?**a

Figure 5 shows the high variability in estimated associations for all three bioactives under investigation. Each estimate shown is based on identical dietary data and thus represents a possible true association between bioactive intake and blood pressure, depending on actual bioactive content. It is noticeable that we observe a Janus-effect with DR-FCT method estimated associations being in opposing positions. This is very noticeable for nitrate, where estimated differences in blood pressure range from -1.0 (95% CI -1.6; -0.4) mmHg between bottom and top decile of intake, suggesting a potentially beneficial, to 0.8 (0.2; 1.4) mmHg, suggesting a potentially detrimental effect on health. As the actual food composition is unknown, it is not possible to obtain a reliable estimate of this association, or even to identify the likely direction of such an association. Using the mean bioactive content, as is common practice, does not resolve this challenge. Biomarker-derived data, while not deprived of limitations but certainly not affected by the factors that modulate variability in food content in DR-FCT method, show a strong and significant inverse association between intake and blood pressure, and this association would have been missed when relying exclusively on dietary data.

These results show that the variability in bioactive content can impact estimated associations between DR-FCT method intake assessments and health endpoints. It demonstrates that even when using the self-same food intake data, differences in bioactive content can result in diametrically opposite results. Considering that most studies investigating associations between the intake of bioactives and health do not take variability in food composition into account, it is likely that many reported associations are unreliable.

Discussion

In this study, we have investigated how the variability in food composition affects nutritional research. Our results, based on three bioactives, show that the variability in food composition represents a significant factor that should be taken into consideration. The use of single point estimates of food composition data represents a significant oversimplification that yields unreliable data as the actual intake can be considerably different from the estimated intake. This is often exacerbated by errors that arise from imputing data into food composition tables from analyses conducted in different countries or by changes in the formulation of foods from food manufacturers. These findings are not only important for observational studies, but also for dietary intervention studies,

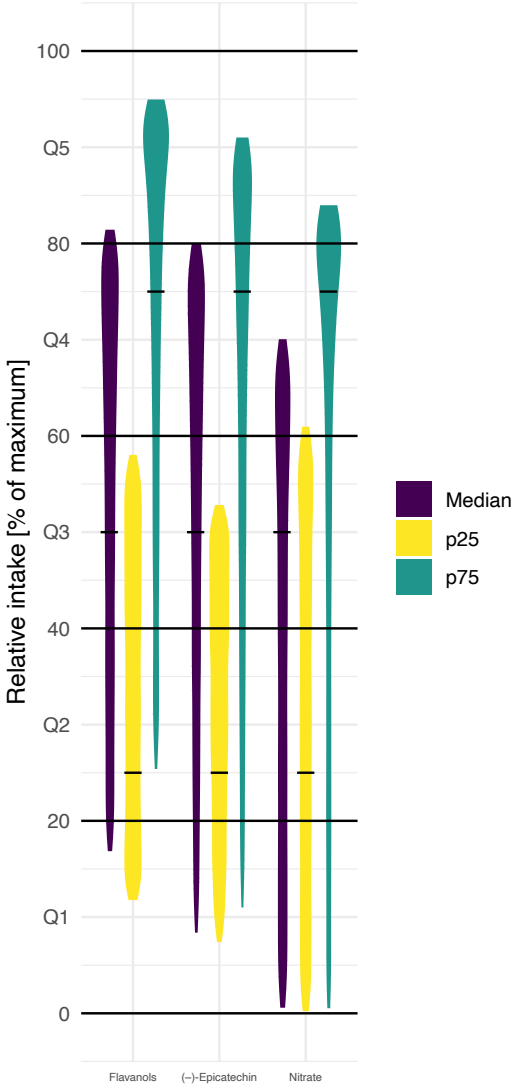

Figure 3. Simulation of the effect of variability in food composition on relative intakes of flavanols, (–)-epicatechin and nitrate of EPIC Norfolk participants with low (25th centile, p25), medium (median) and high (75th centile, p75) estimated intake of bioactive (based on 24HDR and mean food content – indicated by black line). Data shown are relative intake (100% is maximum intake) of 10,000 simulations

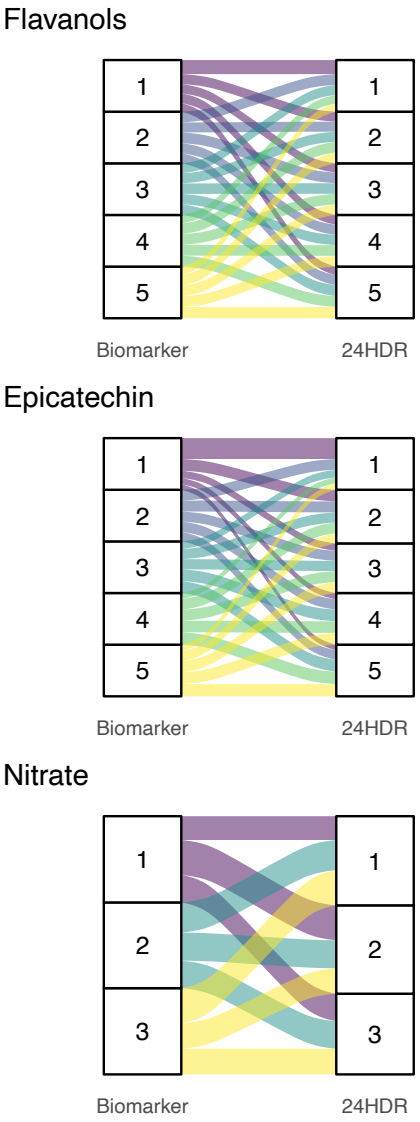

Figure 4. Comparison of biomarker and 24h dietary recall with food-composition data (24HDR – DD-FCT method) estimated quantiles of intake of flavan-3-ols, (–)-epicatechin and nitrate. Bands of the same colour indicate participants in the same biomarker-estimated quantile of intake. 24h dietary recall estimated quantiles of intake were determined the common approach of using mean content of flavan-3-ols, (–)-epicatechin and nitrate in each food item as reported in databases.

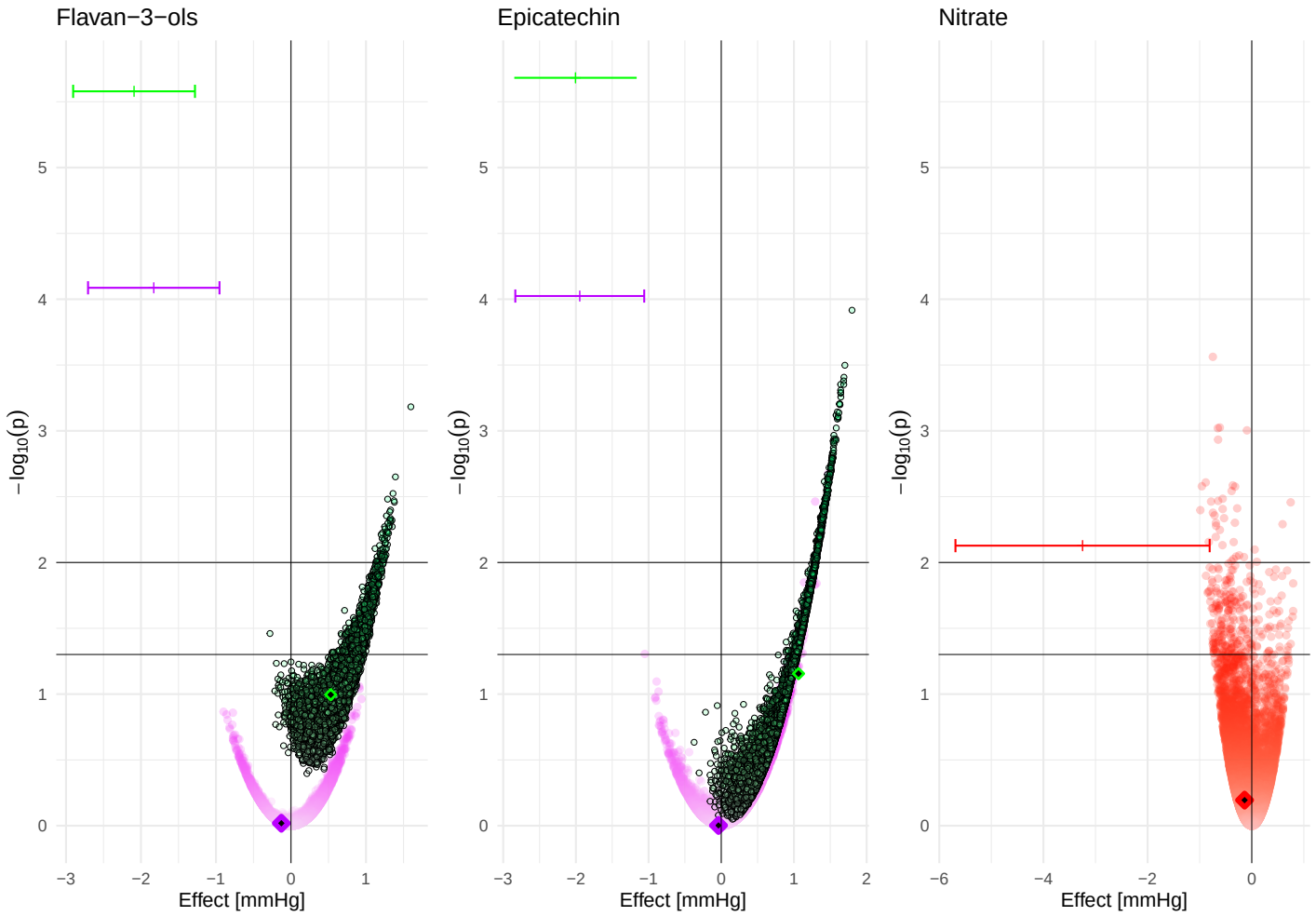

Figure 5. Association between estimated bioactive intake (flavan-3-ols, (–)-epicatechin and nitrate, based on 24h dietary recall and food composition data [DR-FCT method]) and systolic blood pressure at baseline (estimated difference between low [p10] and high [p90] intake and p-value for Wald-test (as -log10(p)) in men (purple), women (green) and all participants (red). Data are based on 10,000 simulations and adjusted for age, BMI, plasma vitamin C, smoking status, physical activity and self-reported health at baseline; additionally for menopausal status for women and sex for nitrate. Results based on intake estimated by simulating food content within minimum and maximum food content reported in databases (circle), intake based on mean food content as reported in databases (diamond) and intake based on biomarker data (|). 95% CI is shown for biomarker only.

where such methods are often used to estimate background dietary intake or even design interventions. The results show that the variability in food composition makes reliable estimates of both, absolute and relative intake of bioactives difficult when solely relying on a combination of self-reported dietary data and food composition databases. This is in addition to the bias introduced by the limitations of dietary assessment. Thus, any associations between intake and health outcomes derived by using this approach are unreliable. Indeed, for the three food constituents under investigation, we found a Janus-like effect with negative and positive associations using the self-same food consumption data and food content within the reported range.

Bioactive intake can only be estimated reliably by using duplicate diets, which can be analysed directly for the compound of interest. A good estimate can also be obtained from properly validated nutritional biomarkers, in particular recovery biomarkers ***Kaaks et al.*** (***1997***), as they are based on the systemic presence of the relevant bioactive compound and do not rely on assumptions about food composition. This is mainly for compounds where food content is very variable, whereas it might be less relevant for constituents of the diet with low variability. These biomarkers are not without their own limitations as they need to be carefully evaluated for their suitability to assess the intake of specific bioactives ***Ottaviani et al.*** (***2018a***, 2019). Most importantly, these biomarkers need to be evaluated using actual food intake and should not rely on published food composition data due to the limitations described. A limitation of nutritional biomarkers is that they require the availability of specimens and that except for recovery biomarkers in 24h urine samples, they can only provide information on relative intake. Moreover, like with 24h dietary recalls, multiple biomarker measurements are required for a more reliable estimate of habitual diet. A further limitation is the known inter-subject variance in biomarkers response to intake due to differences in absorption and metabolism. However, the biomarkers used in this study have been carefully evaluated ***Ottaviani et al.*** (***2018a***, 2019); ***Pannala et al.*** (***2003***) and these data demonstrate a physiological link as well as a strong statistical association between intake and biomarker. Relative intake estimated from these biomarkers is more reliable than that estimated from dietary data.

High variability in food composition has been described for a range of compounds, e.g. for the fatty acid composition of dairy ***Moate et al.*** (***2007***); ***Stergiadis et al.*** (***2019***) or vitamins ***Phillips et al.*** (***2018***). There is also a longitudinal variation in food composition, in particular due to changes to cultivars, production practices, distribution and processing methods ***Davis et al.*** (***2004***), and climate change is likely to exaggerate this ***Macdiarmid and Whybrow*** (***2019***). Thus, bioactive and nutrient content variability must be taken into consideration when choosing the tools to investigate not only dietary bioactives but also micro- and macro-nutrients.

Methods commonly used to address measurement error in nutritional research, such as regression calibration ***Spiegelman et al.*** (***1997***), are not suitable to address this. These methods rely on a known known relationship between reported and actual intake to predict actual intake. However, the composition of the food actually consumed by participants is impossible to predict as it depends on a range of factors, many of which are unknown to consumers and researchers as outlined in the introduction.

There are of course also other sources of bias and variability that affect dietary assessment. We have excluded those from our study as much as possible by using the self-same dietary data for all analyses, using only acute intake data (24h dietary recall and spot urine samples) and an endpoint that is affected directly by intake. This allows us to attribute our findings mainly to the variability in food composition.

In our study, we have used the identical dietary data to investigate the impact of the variability in food content. This has allowed us to exclude other sources of variability in dietary assessment, in particular misreporting of dietary intake. We have also used measures of acute intake (24h dietary recalls and spot urine samples) and used a health endpoint that is directly affected by intake.

Prospective studies

In our study, we have focused on cross-sectional associations between bioactives and blood pressure as the acute effect of these compounds is well established. It is expected that the variability in food composition affects prospective analyses more than cross-sectional analyses: in addition to the variability in food content, the composition of foods changes over time ***Davis et al.*** (***2004***); ***White and Broadley*** (***2005***).

Biomarker-predicted dietary patterns

The high variability in the content of dietary compounds in food has also implications for the development of biomarkers for individual foods or dietary patterns. A number of biomarkers have been proposed to estimate intake of individual foods, for example proline-betaine as biomarker of citrus fruit intake ***Gibbons et al.*** (***2017***), but content in citrus fruits is highly variable (14.3 — 110 mg/100 mL in various citrus fruit juice ***Lang et al.*** (***2017***)) and it is thus not possible to estimate actual food intake without using foods in which the content of the dietary compound to use as a biomarker is standardised.

The same applies to metabolomics-based biomarkers of dietary patterns. They are usually developed under highly standardised conditions and reflect the composition of the foods consumed during these studies. Changes in the composition of these foods affect the concentration of metabolites and thereby reduces the reliability of metabolite-based biomarkers of individual foods or dietary pattern. This diminishes the suitability of such markers for longitudinal or multi-centre studies where a high variability in food composition is likely. These limitations do not apply for the development of biomarkers of specific bioactives or other nutrients as the variability of bioactive and nutrient content are reflected in the variation of biomarker levels.

Effect on dietary recommendations and risk assessment

The findings presented in this work have a considerable impact on dietary recommendations and guidelines. Our data clearly show that results based on DR-FCT method are likely to be biased and unreliable. Dietary recommendation based on such data emanated form that approach are therefore also likely to be unreliable and misleading. However, the high variability in food composition also has an impact on the translation of health-based guidance values into food-based dietary recommendations. For example, the amount of flavan-3-ols required to achieve a vasculoprotective effect according to the EFSA health claim is 200 mg/d ***EFSA Panel on Dietetic Products, Nutrition, and Allergies (NDA)*** (***2014***). When using mean food composition data ***Rothwell et al.*** (***2013***), this could be achieved by 5 cups of tea. However, when using the lowest reported food content, at least 22 cups of tea would have to be consumed to meet recommended intake. Similarly, 5-6 apples would be sufficient to consume the 50 mg/d (–)-epicatechin assumed to be sufficient to improve vascular function ***Ellinger et al.*** (***2012***); ***Hooper et al.*** (***2012***) when using mean food content, but it could be up to 27 when assuming a low content in food. In this manner, it would not be possible to determine whether or not a population is already meeting dietary recommendation for flavan-3-ols unless biomarkers are used for that purpose ***Crowe-White et al.*** (***2022***).

These findings also have an impact on the risk assessment of food components, in particular those that are naturally present in foods and used as additives such as nitrates ***on Food Additives et al.*** (***2017***) or phosphates ***on Food Additives et al.*** (***2019***). Results from observational studies and intervention studies relying on food content data will be affected by inaccurate assessment of intake as described above. More importantly however, the exposure assessment will be affected by the variability of data, with consequences for consumers and food producers as an overestimation of exposure could result in unnecessary restrictions in use, whereas an underestimation could put consumers at risk. For example in EPIC Norfolk, none or only very few study participants exceed the ADI of 3.7 mg/kg BW/d ***on Food Additives et al.*** (***2017***) for nitrate when estimating intake with minimum and mean food content respectively. However, when using the maximum food content, one third of study participants exceed the ADI for nitrate, and almost 10% exceed it by 2-fold. Each

of these scenarios would result in very different actions by risk managers due to the different impact on population health and in the latter case more stringent restrictions were necessary.

Conclusions

These data suggest that the results of many interventional and observational nutrition studies using dietary surveys in combination with food composition data are potentially unreliable and carry greater limitations than commonly appreciated. As these studies are used to derive evidence-based dietary recommendations or risk assessments, their limitations could have a considerable impact on public health as current recommendations might be based on unreliable data. We showed that results relying on food composition data not only failed to identify beneficial associations between three bioactives and blood pressure, but even suggested possible adverse associations. It is likely that findings of this nature are not limited to the model compounds investigated here but apply to other dietary components as well. Given the importance of diet in the prevention of chronic diseases, it is crucial to address this limitation: both by revisiting previous studies and by taking these limitations into consideration in future studies. It is essential to use nutritional biomarkers to determine actual intake to ensure reliable results. This means that the development of more and better biomarkers for accurate dietary assessment remains crucial ***Prentice*** (***2018***). The challenges associated with developing biomarker-based approaches are not insignificant, but the technical capabilities required are broadly available today, and the advantages of deploying improved approaches to establishing links between diet and health are so significant, timely, and needed, that we should make it a standard tool in nutrition research.

Methods

Study population

Between 1993 and 1997, 30,447 women and men aged between 40 and 79 years were recruited for the Norfolk cohort of the European Prospective Investigation into Cancer and Nutrition (EPIC) study, and 25,639 attended a health examination ***Day et al.*** (***1999***). Health and lifestyle characteristics, including data on smoking, social class and family medical history, were assessed by questionnaire. Height and weight measurements were collected following a standardised protocol by trained research nurses. Physical activity, representing occupational and leisure activity, was assessed using a validated questionnaire ***Wareham et al.*** (***2002***). Blood pressure was measured by using a non-invasive oscillometric blood pressure monitor (Acutorr; Datascope Medical, Huntingdon, UK; validated against sphygmomanometers every 6 months) after the participant had been seated in a comfortable environment for 5 minutes. The arm was horizontal and supported at the level of the mid-sternum; the mean of two readings was used for analysis. Non-fasting blood samples were taken by venepuncture and stored in serum tubes in liquid nitrogen. Serum levels of total cholesterol were measured on fresh samples with the RA 1000 autoanalyzer (Bayer Diagnostics, Basingstoke, UK). Plasma vitamin C was measured using a fluorometric assay as described previously ***Khaw et al.*** (***2001***). Spot urine samples were collected during the health examination and stored at -20°C until analysis. The study was approved by the Norwich Local Research Ethics Committee and all participants gave written, informed consent and all methods were carried out in accordance with relevant guidelines and regulations.

Diet was assessed by 7-day diary (7DD), whereby the first day of the diary was completed as a 24-h recall (24HDR) with a trained interviewer and the remainder completed during subsequent days. Diary data were entered, checked and calculated using the in-house dietary assessment software DINER (Data into Nutrients for Epidemiological Research) and DINERMO ***Welch et al.*** (***2001***). Flavan-3-ol intake (the sum of epicatechin, catechin, epicatechin-3-O-gallate, catechin-3-O-gallate and proanthocyanidins) was estimated as described previously ***Vogiatzoglou et al.*** (***2015***); minimum and maximum estimated flavan-3-ol intake was estimated using the minimum and maximum food content data provided by Phenol Explorer und USDA databases ***Rothwell et al.*** (***2013***). Nitrite

and nitrate intake, based on minimum, maximum and mean food content, were estimated using a database published previously ***Blekkenhorst et al.*** (***2017***).

Nutritional biomarker

Flavan-3-ols and (–)-epicatechin

We have used two different biomarkers to estimate flavan-3-ol and (–)-epicatechin intake: $gVLM_B$ that includes the metabolites 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-O-glucuronide (gVL3G) and 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-sulphate (gVL3S), and $SREM_B$ that includes the metabolites (–)-epicatechin-3′-glucuronide (E3G), (–)-epicatechin-3′-sulfate (E3S) and 3′-methoxy(–)-epicatechin-5-sulfate (3Me5S). $gVLM_B$ are specific for estimating the intake of flavan-3-ols in general, including (±)-epicatechin, (±)-catechin, (±)-epicatechin-3-O-gallate, (±)- catechin-3-O-gallate and procyanidins and excluding the flavan-3-ols gallocatechin, epigallocatechin, gallocatechin-3-O-gallate, epigallocatechin-3-O-gallate, theaflavins and thearubigins ***Ottaviani et al.*** (***2018a***). $SREM_B$ are specific for (–)-epicatechin intake ***Ottaviani et al.*** (***2019***). Spot urine samples were collected during the baseline health examination and stored in glass bottles at -20°C until analysis. Stability analyses confirmed that biomarkers are stable under these conditions ***Ottaviani et al.*** (***2019***). Samples were analysed in random order using the method described previously ***Ottaviani et al.*** (***2018a***, 2019), with automated sample preparation (Hamilton Star robot; Hamilton, Bonaduz, Switzerland). Concentrations below the lower limit of quantification (LLOQ, 0.1 μM) were used for the analysis to avoid the bias of substituting a range of values by a single value. Concentrations were adjusted by specific gravity for dilution as the endpoint of the analysis, systolic blood pressure, was strongly correlated with urinary creatinine. We have used specific gravity to adjust for dilution previously when there was a strong association between creatinine and study endpoint ***Bingham et al.*** (***2007***).

Flavan-3-ol and (–)-epicatechin biomarker data, as well as data for all other variables were available for 18,864 participants. Data for nitrate biomarker were available for 1,027 participants.

Nitrate

Urinary nitrate concentration, adjusted for dilution by specific gravity, was used as biomarker of nitrate intake, as between 50% and 80% of dietary nitrate are recovered in urine, whereas endogenous production is relatively stable at 0.57 (95% CI 0.27 – 0.86) mmol/d ***Green et al.*** (***1981***); ***Packer et al.*** (***1989***). A random subset of 1,027 samples were analysed by ion chromatography with colorimetric detection (NOx Analyser ENO-30, EICOM, San Diego, CA).

Simulation of variability

We have conducted 10,000 simulations to explore the impact of the variability in bioactive content. For each simulation, we assigned each participant a possible intake of total flavan-3-ol, (–)-epicatechin and nitrate based on their self-reported dietary intake and the minimum and maximum reported content of each compound in the foods consumed. The data available do not suggest that food composition follows a normal distribution, and we have therefore assumed a uniform distribution.

Data analysis

Data analyses were carried out using R 3.6 ***R Core Team*** (***2023***), using the packages rms ***Harrell Jr*** (***2023***) for regression analyses, ggplot2 ***Wickham*** (***2016***) and gridExtra ***Auguie*** (***2017***) for the generation of graphics. Regression analyses were conducted using `ols` as regression function. We have used the Wald statistics calculated by the *rms* `anova` function to investigate the relationship between dependent and independent variables, and to test for linearity. tableone ***Yoshida and Bartel*** (***2022***) was used to prepare tables. Unless indicated otherwise, results are shown with 95% confidence intervals.

Descriptive statistics

Descriptive characteristics of the study population were summarised using mean (standard deviation) for continuous variables and frequency (percentage) for categorical variables.

Data transformation

Biomarker data were positively skewed (log-normal distribution) and therefore log2-transformed data were used for all analyses. Restricted cubic splines (3 knots, outer quantiles 0.1 and 0.9; using the rcs function ***Harrell Jr*** (***2023***)) were used for all continuous variables unless indicated otherwise.

Cross-sectional analyses

In cross-sectional analyses, stratified by sex, we investigated associations between biomarker and 24h recall estimated flavan-3-ol, (–)-epicatechin and nitrate intake (biomarkers adjusted by specific gravity adjusted, dietary data by energy, log2-transformed), as independent variable and systolic and diastolic blood pressure [mmHg] using multiple regression analyses. Analyses were adjusted by age (continuous; years), BMI (continuous, kg/m^2), plasma vitamin C, smoking status (categorical; never, ever, former), physical activity (categorical; inactive, moderately inactive, moderately active, active) and health at baseline (self-reported diabetes mellitus, myocardial infarction, cerebrovascular accident). Analyses with flavan-3-ol and (–)-epicatechin as independent variable were stratified by sex, and analyses for women additionally adjusted by menopausal status; analyses with nitrate as independent variable were adjusted by sex and menopausal status.

Availability of data and code

Data from the EPIC-Norfolk study must be requested directly from their data request team by completing a data request form. Code will be available on request from the authors.

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Declaration of comepting interest

HS and JIO are employed by Mars, Inc. a company engaged in flavanol research and flavanol-related commercial activities. GGCK has received unrestricted research grants from Mars, Inc.

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