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Article

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TITLE

# An analytical pipeline for quantitative characterization of dietary intake: application to assess grape intake

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## Abstract

Lack of accurate dietary assessment in free-living populations requires discovery of new biomarkers reflecting food intake qualitatively and quantitatively to objectively evaluate effects of diet on health. We provide a proof-of-principle for an analytical pipeline to identify quantitative dietary biomarkers. Tartaric acid was identified by Nuclear Magnetic Resonance spectroscopy as dose-responsive urinary biomarker of grape intake and subsequently quantified in volunteers following series of 4-day dietary interventions incorporating 0g/day, 50g/day, 100g/day and 150g/day of grapes in standardized diets from a randomized controlled clinical trial. Most accurate quantitative predictions of grape intake were obtained in 24h urine samples which have the strongest linear relationship between grape intake and tartaric acid excretion ($r^2$=0.90).

This new methodological pipeline for estimating nutritional intake based on coupling dietary intake information and quantified nutritional biomarkers was developed and validated in a controlled dietary intervention study, showing that this approach can improve accuracy of estimating nutritional intakes.

**Key words:** accurate dietary assessment, metabolic profiling, nutritional intake, quantified dietary biomarkers, tartaric acid

## INTRODUCTION

Higher consumption of vegetables and fruits is associated with lower all-cause mortality and recent studies suggest that daily intakes of 5 (*1*) or 7 or more portions of vegetables and fruits (*2*) lowers the risk of death, particularly cardiovascular mortality. Such evidence is the basis for government recommendations to improve eating patterns. However, monitoring compliance to dietary advice at the population level is extremely difficult because existing dietary assessment tools based on self-reporting methods are inherently inaccurate (*3, 4*). Under-reporting was found to be 34% in men and 33% in women, with the highest occurrence of under-reporting in obese and overweight individuals (*5, 6*). There is an unmet need for quantifiable dietary biomarkers that accurately reflect consumption of foods and nutrients (*4*).

Dietary intake biomarkers are based on the concept that excretion levels of food-related metabolites are highly correlated with food intake over a given period of time. These biomarkers can be components of the food itself, excreted unchanged or compounds that have undergone metabolic conversion by the human or by the resident gut bacteria. Global metabolic profiling using spectroscopic technology has been applied to detect food-derived compounds (*7*) including polyphenols (berries) (*8*), alkyl resorcinols (wheat) (*9*), proline betaine (citrus fruit) (*10*), (*N*-acetyl-)*S*-methyl-*L*-cysteine sulfoxide (cruciferous vegetables) (*11*), and trimethylamine-*N*-oxide and methylhistidine (oily fish) (*8*) which are potential biomarkers of original food intake. Metabolic profiling allows an independent and objective assessment of food intake from which energy and nutrients can be calculated. Whilst much of this metabolic profiling research was conducted using panels of metabolites or excretion patterns, few studies have established quantitative relationships between amounts of specific foods consumed and food-derived metabolites excreted. Exceptions include: total

urinary nitrogen for protein intake, urinary potassium and sodium outputs for potassium and sodium intake (*12*) and proline betaine derived from citrus fruit consumption which exhibits total clearance within 24h (*13*). However, as with many putative biomarkers of individual foods, proline betaine is not entirely specific to citrus fruits and can be found in low concentrations in alfalfa, pulses, kiwi and pears (*10, 14*). Nevertheless, proline betaine has been shown to be a robust and quantifiable dietary biomarker of citrus intake (*10, 13*).

Specific foods may have benefits for specific diseases. For example, consumption of grapes and grape-based products has potential efficacy in cancer prevention (*15-18*) and is associated with decreased risk for cardiovascular disease (*19*), however there is no irrefutable evidence for the health benefits of grapes from randomized controlled intervention studies. A methodology for generating reliable markers of specific foods such as grapes would be a valuable addition to the armory of nutritional tools. Here we develop and apply a rapid, accurate and efficient analytical pipeline for assessing food intake using Nuclear Magnetic Resonance (NMR) spectroscopy and demonstrate its application in measuring urinary concentrations of tartaric acid as an indicator of grape intake.

## MATERIALS AND METHODS

### Identification of a candidate biomarker for grape intake

To explore potential quantifiable biomarkers of intake of grapes, we designed a grape challenge pilot study that was undertaken to characterize the urinary metabolite excretion profile following consumption of grapes. Six volunteers (3 men and 3 women, age range: 22-32, BMI range: 21.2-25.3 $kg/m^2$) were recruited and participated in a 3-day study. Participants were healthy, non-smokers and did not consume drugs or food supplements regularly. The grapes were administered as part of a standard breakfast consisting of one cup of tea or coffee and a fruit salad containing apple, pineapple and red grapes. The amount of red grapes in the consumed fruit salad was increased over the three consecutive days viz. 50g, 100g and 200g of red grapes on days 1, 2 and 3, respectively, whilst apple and pineapple intake remained constant. For the 24h preceding the grape challenge and throughout the remaining part of each experimental day, participants ate their habitual diet avoiding any products containing grapes, raisins or food products derived from grapes. All urine produced was collected daily into four timed aliquots per day corresponding to 0-4h, 4-8h, 8-12h and 12-24h post consumption, using single-use urine containers (International Scientific Supplies Ltd, Bradford, United Kingdom). In addition, a spot urine sample was collected on day 1 prior to consumption of grapes to provide a baseline profile. Urine samples were stored at -80°C until analysis.

### Compositional analysis of grapes study

To characterize the chemical composition of varieties of grapes consumed in the UK, ten varieties of red grapes and two varieties of green grapes from five countries were selected according to seasonal availability. Two batches of 400g of each variety of grapes were purchased and for each variety 50g of grapes were picked from random

areas of different clusters to provide a representative sample. A total of three replicates of each batch were prepared. Each individual sample was homogenized using a Kenwood KMix Blender for 5min and, approximately, 50ml of must were obtained and then filtered using a stainless steel filter. An aliquot of 1ml of each sample was centrifuged for 5min at 16,000xg, and the supernatant fluid was analyzed by $^{1}$H-NMR spectroscopy. Quantification of the tartaric acid concentration in red and green grapes was carried out using a standard one-dimensional NMR pulse sequence ensuring fully relaxed pre-saturation of the water resonance.

**$^{1}$H-NMR spectroscopic analysis of urine and grape samples**

An aliquot of each urine sample (540µl) was mixed with 60µl of pH 7.4 phosphate buffer containing trimethylsilyl-[2,2,3,3,-$^{2}$H4]-propionate (TSP) as an internal chemical shift reference before being prepared for the NMR spectroscopic analysis following the protocol described by Dona *et al.* (*20*). Urine samples were analyzed in 96-well plates containing one quality control (QC) sample every ten samples. QC samples were prepared by pooling 50µl of each urine sample. Filtered homogenates (400µl) of representative samples of the grapes given to participants were mixed with 200µl of pH 7.4 phosphate buffer. QC samples of grape homogenates were prepared by pooling 50µl of each grape fluid sample and analyzed every ten samples.

$^{1}$H-NMR spectroscopy was performed at 300K on a Bruker 600MHz spectrometer (Bruker BioSpin, Karlsruhe, Germany) using the following standard one-dimensional pulse sequence with saturation of the water resonance: RD–gz,1–90°–t–90°–$t_m$–gz,2–90°–ACQ, where RD is the relaxation delay, t is a short delay typically of about 4µs, 90° represents a 90° radio frequency (RF) pulse, $t_m$ is the mixing time (10ms), gz,1 and gz,2 are magnetic field z-gradients both applied for 1ms, and ACQ is the data acquisition period (2.7s). Water suppression was achieved through continuous wave

irradiation at the water resonance frequency using 25Hz RF strength during RD and also during $t_m$. The receiver gain was set to 90.5 for all experiments. Each urine spectrum was acquired using 4 dummy scans, 32 scans, 64K time domain points and with a spectral window set of 20 ppm. Prior to Fourier transformation, the free induction decays were multiplied by an exponential function corresponding to a line broadening of 0.3Hz. To achieve accurate quantification of metabolites, it was necessary to ensure that both tartaric acid and TSP resonances were fully relaxed using a long RD ($7\times t_1$) between each pulse. The inter-pulse delay time $d_1$ was therefore set to 100s (*21*).

**Pre-processing of NMR spectra**

The $^1$H-NMR spectra were digitized over the range of δ10.0 to -0.5 and imported into MATLAB (2014a, Mathworks Inc., USA), and automatically phase- and baseline-corrected. Urine spectra were then referenced to the internal standard, TSP at δ0.0. The spectral regions occupied by water and urea (δ4.45−6.95) and TSP (<δ0.35) were excluded. Each spectrum was normalized to the total urine volume excreted in order to correct differences in urinary dilution. Spot urine samples were normalized to the spot volume. Each spectrum of grape juice extract was phased and baseline corrected as above, and normalized to the TSP signal.

**Statistical analysis**

Urinary global profiling analysis in combination with unsupervised Principal Component Analysis (PCA) (*22*) and supervised Partial Least Squares Discriminant Analysis (PLS-DA) (*23*) was applied to identify candidate biomarkers of grape intake. PCA was used to visualize any trends in the data and these trends were then further analyzed using PLS-DA and Monte-Carlo cross-validation (MCCV) (*24*). For each component in the PCA model the percentage of explained variance ($R^2_X$) was

calculated. The variability of the predicted scores was visualized using Kernel Density Estimation (KDE). Across all MCCV models, the mean score and variance of the score for each sample were used to calculate the KDE. Specifically, for each sample the mean and standard deviation across all MCCV models can be used to give a normal distribution of the predicted scores. Taking the sum of all individual distributions within each class then yields the KDE as shown in the figures. The stability (variance) of each regression coefficient was assessed across the MCCV models using bootstrap resampling (*25*) of each model. Using the variance and mean regression coefficient, a t-score, and subsequently a *P*-value, were calculated. The *P*-values were corrected for multiple testing using the Storey-Tibshirani (*26*) False Discovery Rate (q-value). The goodness of fit ($R^2_Y$) of the MCCV models was calculated across all models using the training data and the goodness of prediction ($Q^2_Y$) for the test data.

Hierarchical cluster analysis (HCA) was used to investigate correlations among the identified biochemical components of different variaties of grapes, specifically to determine whether the concentration of tartaric acid shows similarity with other compounds. To quantify the relationship, HCA was used to determine clustering in the data. Significance of the correlation between pairs of compounds was assessed based on a Bonferroni correction to the *P*-values. HCA was applied to the resulting correlation matrix and the optimal number of clusters was determined by calculating the modularity (*27*) of the network and choosing the highest modularity as optimal number of clusters. The modularity is a weighted measure between the number of links (correlations) within a cluster and the number of links from one cluster to other clusters, with clusters defined by cutting at different heights of the hierarchical clustering tree. The highest modularity indicates that, relatively, there are more within-cluster links compared to between-cluster links.

Standard linear regression was used to relate grape intake to tartaric acid excretion. The variability of the regression coefficient is visualized in corresponding plots as the 95% confidence intervals (CI) estimate by bootstrap resampling of the regression coefficient. The squared correlation coefficient of the model ($r^2$) is a measure of linearity of the data and corresponding model. Bland-Altman (*28*) plots were used to show the agreement between the estimated intake of grapes versus the real intake of grapes.

PCA and PLS-DA analyses were also carried out on spectral data of grape homogenates normalized to volume in MATLAB to compare different grape varieties. Correlation of spectral variables using Statistical Total Correlation Spectroscopy (*29*) and Subset Optimization by Reference Matching (*30*) were used as data-driven approaches to aid metabolite identification. Confirmation of metabolite identities was obtained using 1D and 2D NMR experiments (spiking of chemical standards, J-Resolved spectroscopy, Total Correlation Spectroscopy, Hetero-nuclear Single Quantum Coherence spectroscopy).

**Quantification of tartaric acid related to grape intake**

Nineteen volunteers (10 male and 9 female, age range: 25-60, BMI range: 21.1-33.3kg/$m^2$) attended the NIHR/Wellcome Trust Imperial CRF for four 4-day inpatient periods separated by a period of >3 days. Potential subjects were excluded if they had clinically significant illnesses, were taking prescription medication, current smokers, a history of substance abuse, and any abnormalities detected on physical examination, electrocardiography, or screening blood tests (measurement of complete blood count, electrolytes, fasting glucose and lipids, thyroid function and liver function). Women were ineligible if they were pregnant or breast-feeding. In a random order, participants were provided with four different diets throughout each of the 4-day inpatient periods.

Each of the four diets represented 25% (diet 1), 50% (diet 2), 75% (diet 3) and 100% (diet 4) of the healthy eating targets based on UK recommendation for fruits, fats, sugars, vegetables, carbohydrates, fiber and salt. Grapes were consumed as an afternoon snack two hours after lunch. Alcohol and grape derived products were not provided as part of any of the four diets.

The amounts of red grapes administered daily were 0g (diet 1), 50g (diet 2), 100g (diet 3) and 150g (diet 4) (**Figure 1**). The randomization procedure was conducted by an investigator not directly involved in the study with the use of opaque, sealed, sequentially numbered envelopes that each contained a random order for the four dietary interventions. The envelopes were stored securely and opened in sequence by an investigator (ESC) once volunteers had been recruited. Volunteers and investigators could not be blinded during data collection; however, all investigators conducting data analysis were blinded to the randomization order. Different varieties of red grapes from different countries were provided according to seasonal availability. Wine, raisins and any fruit juice were excluded from the experimental diets. Moreover, volunteers did not take any supplements and minimal physical activity was imposed. Fasting spot urine samples were collected on arrival at the NIHR/Wellcome Trust Imperial Clinical Research Facility and daily thereafter for the four days of each of four dietary interventions. Each participant collected cumulative urine samples (CS) daily, over the four-day period of each dietary intervention, from after breakfast to before lunch (CS1), from after lunch to before dinner (CS2) and from after dinner to next day fasting urine sample (CS3). Finally, a 24h urine sample was obtained by pooling CS1, CS2 and CS3. In addition, a spot sample was collected daily two hours after the afternoon snack. Aliquots of urine were transferred into Eppendorf tubes and stored at -80$^{o}$C until analysis by $^{1}$H-NMR spectroscopy. All subjects provided informed, written

consent prior to the clinical trial (Registration No: ISRCTN 43087333), which was approved by the London Brent Research Ethics Committee (13/LO/0078). All studies were carried out in accordance with the Declaration of Helsinki.

**Monitoring in-patient volunteers for 24h tartaric acid excretion**

Samples from day 3 of each dietary intervention were chosen to monitor volunteers' excretion (Figure 1) of tartaric acid over 24h, as well as in the fasting urine sample of day 3 of the study, spot urine sample collected 2h after grapes consumption and the fasting urine sample on the day 4 of the study.

Tartaric acid gives rise to a single peak in the NMR spectrum at δ4.34. This signal was integrated for the urine samples using an automated algorithm (*31*). The amount of tartaric acid excreted in 24h was calculated by dividing the corresponding integral by the number of $^{1}$H of tartaric acid signals (4×$^{1}$H) and multiply with the number of $^{1}$H in TSP (9×$^{1}$H). Then multiplying with the concentration of TSP in the sample gives the concentration of tartaric acid. Three calibrations curves corresponding to CS2, CS3 and 24h urine samples, were built to establish the relationship between excretion of tartaric acid and the amount of grapes consumed.

**Prediction of grape intake based on tartaric acid urinary excretion in a randomized highly controlled clinical trial**

Tartaric acid was quantified for each of the spot and cumulative sample sets (corresponding to 0-3h post consumption (CS2); 3-15h post consumption (CS3) and the 24h urine cumulative samples) for each of the four levels of grape intake (0, 50, 100, 150g) to investigate how accurately grape intake could be estimated in a highly controlled clinical trial. The models were trained using calibration curves built using the tartaric acid signals from urine spectra derived from samples obtained on the third day of the study (n=304). The model was then used to predict the quantity of grape intake

236 using samples collected on the first and the second day of each dietary intervention
237 (n=608).

## RESULTS AND DISCUSSION

### Monitoring $^{1}$H-NMR urinary global profile over 24h following grape intake

The tartaric acid signal corresponding to a singlet at δ4.34 was identified from the global profiling analysis of the pilot human intervention study (n=6) as a candidate marker of grape consumption. The signal for tartaric acid was absent in the baseline urine sample prior to grape consumption and showed an incremental increase in intensity as the amount of grapes consumed increased. Although other signals from metabolites such as glucose, hippurate and 4-hydroxyhippurate were also qualitatively associated with grape intake, the tartaric acid signal was the only peak in the $^{1}$H-NMR global metabolic profile observed to increase proportionally with incremental grape intake (**Figure 2**A) over the three consecutive days in all participants. The urinary excretion kinetics of tartaric acid was calculated. In all participants, excretion of tartaric acid peaked between 4h and 8h post-intervention and the majority of the excretion occurred in the first 12h. Tartaric acid concentrations declined almost to baseline after the 12-24h collection.

### $^{1}$H-NMR global profiling of red and green grapes

To confirm the dietary origin of tartaric acid we obtained 96 $^{1}$H-NMR global profiles of red (n=63) and green grapes (n=33) from different varieties and countries (as listed in Figure 2B). As expected, tartaric acid was one of the dominant compounds from the 31 metabolites identified in the global metabolic profiles of grapes (**Supplementary Figure 1**). The PCA scores plot showed clustering of grapes according to variety and country (Figure 2B) with the Black Princess (Chile) and the Sharad (India) varieties being most distinctive in profile. Moreover, the PCA model including all the red and green grape samples showed a trend in clustering according to the color of the grape (Figure 2C). Systematic differences between red and green grapes were determined

from the PLS-DA model ($R^2_Y$=0.65, $Q^2_Y$=0.52) (**Figure 3**A, B), including significantly higher concentrations of phenylalanine and leucine in green grapes whereas ethyl glucuronide was significantly higher in the red grapes (Figure 3C). The concentration of tartaric acid (Figure 3C, inset) was not significantly different between red and green grapes or varieties indicating that it could have general applicability as a marker of grape intake.

Correlations between the 31 small molecules displayed in a heat-map (Supplementary Figure 1) showed grouping of chemical components of the grape homogenate. Examples of distinct clusters, correspond to amino acids in one and for instance ethanol, acetoin and 2,3-butanediol (markers of fermentation) in another cluster. It shows that tartaric acid is an independent component in grapes as it is independent of other small molecules found in grapes.

**Assessment of tartaric acid as a quantitative biomarker of grape intake in human urine samples**

As expected, the third and fourth day spot-fasting urine samples and the CS1 urine sample (cumulative sample from after breakfast to before lunch) collected during the controlled clinical trial did not contain any tartaric acid as the samples were collected before volunteers ingested red grapes (afternoon snack).

The calibration curves for tartaric acid calculated using urine samples obtained on the third day of each dietary intervention showed a linear relationship between quantity of grapes consumed and tartaric acid excreted, with the exception of the spot urine samples. Although, spot urine samples collected 2h after grape intake showed some evidence of tartaric acid excretion, there were no significant differences in concentrations (**Figure 4**A) in relation to the different amounts of grapes consumed ($r^2$=0.04). The CS2 urine samples (collected from after lunch to before dinner) (Figure

4B), which contained the urine excreted in the first three hours following consumption of grapes as an afternoon snack, showed a linear relationship with a correlation coefficient of $r^2$=0.58.

The correlation coefficient defining the relationship between amount of grape intake and tartaric acid excretion corresponding to samples that were collected overnight (CS3: 12h collection from after dinner to next day fasting urine sample) was stronger ($r^2$=0.80) than the correlation coefficient (Figure 4C) found for the previous period (CS2). However, the 24h calibration curve (Figure 4D), showed the highest correlation coefficient $r^2$=0.90 and therefore it was used to quantify the total 24h urinary tartaric acid excretion for day three. On average, 0.16, 0.30 and 0.49mMol of tartaric acid was excreted in 24h urine samples after eating 50, 100 and 150g of grapes, respectively. The mean, standard deviation and 95% confidence interval of urinary tartaric acid excreted calculated for the three urine collection period: 24h (0.161±0.035mMol (0.101-0.231)), CS3 (0.115±0.040mMol (0.026-0.195)) and CS2 (0.046±0.023mMol (0.014-0.093)) samples after eating 50g of grapes.

**Calculation of the ratio of tartaric acid consumed and tartaric acid excreted in the urine**

The mean concentration of tartaric acid in 50g of red grapes (ten different varieties) and in 50g of green grapes (two different varieties) was 0.84±0.03mMol for red and 0.85±0.08mMol for green grapes (**Supplementary Table 1**). These results corroborated the PLS-DA results, which found no significant differences in the quantity of tartaric acid according to grape color or variety. The amount of tartaric acid excreted unchanged in the urine after consumption of 50g of red grapes represented 19.2% of the amount consumed. Of this 5.5% was excreted in the first 3h post consumption (CS2) and the remaining 13.7% was excreted 3-15h post consumption (CS3).

**Estimation of grape intake based on tartaric acid urinary excretion in a randomized highly controlled clinical trial**

Finally, we assessed the quantity of grapes consumed during the controlled clinical trial based on the models calculated from tartaric acid excretion in the urine samples collected on day 3. The CS2, CS3 and 24h urine samples collected during the first and the second day of the control clinical trial were used as a test set of samples for this purpose. The amount of urinary tartaric acid excreted was interpolated from the calibration curve built with the CS2, CS3 and 24h urine samples obtained on the third day of the study in order to estimate the amount of grapes consumed by the volunteers on the first two days in each intervention.

The Bland-Altman plots of the actual and estimated grape intake in CS2, CS3 and 24h samples are shown in **Figure 5**A, B and C, respectively. These plots highlight that the prediction of mean grape intakes in grams were close to the known intakes of 50g (50.9g), 100g (91.4g) and 150g (163.0g) of grapes and also verify that tartaric acid was not present in diet 1 where grapes were not consumed. We found that accuracy of the predicted intake was inversely proportional to the amount of grapes consumed for all collection periods (CS2, CS3 and 24h). In general, the most accurate prediction of intake was found in the 24h urine sample (Figure 5) indicating that collection of 24h samples is the most appropriate strategy for estimation of grape intake.

## Development of a quantifiable biomarker for grape consumption

There is a need to develop quantifiable dietary biomarkers for a range of food and nutrients to identify healthy and/or unhealthy eating patterns, since examples of validated quantifiable nutritional biomarkers are rare. Development of measurable biomarkers of intake of specific foods in body biofluids presents a complex challenge and is a multistage procedure involving: *i)* discovery and identification of chemical(s) reflecting exposure to specific dietary components, *ii)* assignment of candidate biomarkers to endogenous or exogenous origin, *iii)* validation of candidate biomarker in an independent cohort, *iv)* evaluation of the most appropriate biological sample for quantification of the biomarker and *v)* investigation of the specificity and sensitivity of the proposed nutritional biomarker with respect to estimating exposure to specific food or food groups in an epidemiologic context.

In the current study, we developed a quantifiable biomarker for grape intake. $^{1}$H-NMR spectra of urine specimens from a pilot study showed tartaric acid to be a candidate biomarker reflecting exposure to incremental grape consumption. The follow-up kinetic study showed that the majority of tartaric acid was excreted between the first 4h and 8h post-intervention in all participants, with almost complete excretion within 24h post-consumption (Figure 2A). The dietary origin of tartaric acid as natural organic acid in grapes at high concentrations is well known. It is also present in lower amounts in bananas, cranberries and tamarinds, but rare in most other common plants (*32, 33*). Traces of tartaric acid can also be found in processed foods as an acidifying agent (*34*). PCA analysis of red grapes showed clustering according to variety and country (Figure 2B), which has been demonstrated due to soil, climatic region, and cultivar practices (*35*) and is reflected in the variation of sugars, amino acids and organic acids. However, differences in chemical composition reflected in PCA and PLS-DA

models were unrelated to tartaric acid excretion. Tartaric acid was found in all varieties of grapes tested, and the concentration was similar between varieties (Supplementary Table 1). Thus it is feasible to suggest that tartaric acid serves as a robust, NMR-quantifiable biomarker of grape intake, independent of origin and grape variety.

**Evaluation of optimal sampling strategy**

We evaluated the most appropriate sampling strategy for detection and quantification of dietary biomarkers based on 24h total urinary biomarker excretion vs. cumulative timed and spot urine collections. The evaluation was conducted by monitoring 24h urinary excretion of tartaric acid in volunteers attending a highly controlled dietary intervention study. Findings unequivocally indicated that the cumulative 24h collection performed best in terms of the ability to accurately estimate the quantity of grapes consumed and to detect and quantify tartaric acid. Evaluation of spot urine samples, as a more practical alternative in terms of study cost and participant compliance, indicated that it was not possible to obtain accurate estimates of grape consumption from spot urines obtained either at 7am following an overnight fast or 2h after grape intake. Although there was evidence of urinary excretion 2h after grape intake in the spot urine samples, there was no strong association between dose and amount of tartaric acid excreted. This can most likely be explained by inter- and intra-individual variability in human metabolism. Tartaric acid is an exogenous compound, the majority of which is not absorbed. Our results concur with previous human metabolism studies suggesting that 15-20% of the dietary tartaric acid is excreted in the urine unchanged (*36, 37*). Tartrate either undergoes bacterial digestion in the large intestine, by at least 23 varieties of bacteria, or it is excreted in the stool in the form of an insoluble salt such as calcium tartrate (*36*), which can affect the ratio of tartaric acid excreted in feces vs. urine and explain inter-individual differences in excretion.

However, cumulative urine samples CS2 and CS3 showed a linear relationship between grape intake and tartaric acid urinary excretion. These results emphasize the importance of understanding the kinetics of biomarker excretion in order to select the best time and sampling strategy, particularly if spot urine samples are to be used to detect specific dietary biomarkers. The optimal sampling time should correspond to the peak excretion time, which will be specific for each dietary biomarker. For example, proline betaine is predominantly detected between 2-6h after citrus intake (*10*), whilst the optimal window for tartaric acid was between 4-8h after grape intake. With this in mind, nutritional epidemiological studies applying global metabolic profiling strategies should collect the first spot urine sample in a 3-6h time window after the meal or food challenge. It is crucial that urine is collected at the same time for each study participant to avoid introduction of unnecessary variation. Consideration should be given as to whether the biomarker is unique to a given food: for example tartaric acid is itself added as a preservative to some processed foods and beverages. Moreover, it is important to note that sample preservatives can react with biomarkers an example being the complex tartaric acid forms with boric acid (*38*) altering the intensity and shape of the peak. As expected, 24h urine samples remain the best option for total quantification of tartaric acid as a dietary biomarker since total excretion occurs in this time period regardless of inter-individual differences.

**Limitations in the proposed strategy for the quantification of dietary biomarkers**

The major confounder for assigning individual chemicals as biomarkers of specific foods or nutrients is the fact that these candidate biomarkers are seldom unique to a specific food. Urinary tartaric acid, in addition to being a major component of grapes, is also found in high concentrations in grape juice and wine, whilst lower concentrations can be found in other fruits or as additives. We found that 19.2% of all

tartaric acid present in 50g of grapes is excreted in the urine unchanged, therefore over 80% of tartaric acid is metabolized to other compounds. However, to address the lack of specificity of biomarkers, one solution to for instance differentiate grape and wine-derived tartaric acid is to utilize biomarker patterns to ascertain dietary origin. Biomarkers of wine intake identified from interventional studies include gallic acid, 4-o-methyl gallic acid (*39*), caffeic acid and catechins. Of these, resveratrol is the only wine intake biomarker validated in clinical and epidemiological studies (*40, 41*). Therefore, resveratrol could be used to estimate the amount of tartaric acid expected to derive directly from wine, and will allow the differentiation of urinary tartaric acid excreted as a consequence of grape intake or wine intake or both. We found the amount of tartaric acid to be comparable between red and white grapes, therefore it is a stable biomarker for grapes. However, it has been shown that resveratrol is found in higher concentrations in red and white grapes and has been validated as biomarker of red wine (*34*). In the case of intake of both red grapes and red wine, assessing the ratio between resveratrol and tartaric acid excreted in urine following wine intake alone is needed in order to determine the amount tartaric acid that comes from wine and grapes. Ethanol and ethylglucuronide can also be used to assess consumption of alcoholic beverages in general to supplement using resveratrol alone.

Despite the fact that many studies have focused on the identification of wine (*40, 41*) or grape juice (*42*) biomarkers, we are not aware of any studies reporting (quantitative) biomarkers of grape intake to date. The present work applies a new analytical pipeline to assess tartaric acid as a quantitative biomarker of grape intake that could be used in clinical and epidemiological studies in order to assess accurate grape intake. Grapes are rich in polyphenols, which contain antioxidants, conferring health benefits such as reduced risk of certain cancers. Dietary health policies are

based on traditional dietary assessment methods, which are prone to misreporting. We propose a strategy using NMR as an alternative to current dietary reporting methods and demonstrate its applicability in a controlled nutritional trial. However this strategy can be extendable to other analytical techniques in order to quantify other types of compounds.

In summary, we developed an analytical pipeline employing multivariate statistics for identifying a candidate food intake biomarker, followed by calculation of urinary concentration from $^{1}$H-NMR signal intensities and estimating grape intake using calibration curves. We describe a new dietary assessment tool that can be used to confirm intake of specific foods. We exemplified this approach using tartaric acid as a quantifiable biomarker of grape intake. Although this strategy was developed in the context of a highly controlled dietary intervention study, we provide proof-of-principle that coupling self-recorded dietary intake information with quantified nutritional biomarkers may be used to achieve a more objective measure of dietary exposure. Joining efforts from the nutritional and epidemiological researches identifying as many quantitative dietary biomarkers as possible will allow us in future to accurately assess dietary intake both individually and in a population framework. Further studies are required to validate the application of this strategy to assess accurate dietary intake in free-living people.

**ABREVIATIONS**

BMI: Body Mass Index; CI: Confidence Interval; CS: Cumulative (urine) Sample; $^{1}$H: proton; HCA: Hierarchical Cluster Analysis; KDE: Kernel Density Estimation; MCCV: Monte-Carlo Cross Validation; NMR: Nuclear Magnetic Resonance; PCA: Principal Component Analysis; PLS-DA: Partial Least Squares Discriminant Analysis; QC: Quality Control; $Q^2_Y$: goodness of prediction; $R^2_Y$: goodness of fit; $r^2$: squared

457 correlation coefficient; RD: Relaxation Delay; RF: Radio-Frequency; $t_m$: mixing time;
458 TSP: trimethylsilyl-[2,2,3,3,-$^2H_4$]-propionate.

459 **ACKNOWLEDGMENTS**

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461 **SUPPORTING INFORMATION**

462 Supplementary Figure 1: Correlation of 31 identified compounds in $^1$H-NMR global
463 profiles of red and green grapes visualized in a heat map with hierarchical clustering
464 applied.

465 Supplementary Table 1: Mean values of quantified tartaric acid in different varieties of
466 red and green grapes from different countries.

467 **AUTHOR INFORMATION**

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470 **Author contributions**

471 IGP, JMP, EH and GF designed research; IGP and ESC conducted the clinical trial;
472 IGP conducted research; JMP and IGP analyzed data and performed statistical
473 analyses; IGP, JMP, JKN, JCM, JD, EH and GF wrote the paper. GF had final
474 responsibility for final content. All authors read and approved the final manuscript.

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## Notes

GF has personal links with the food industry through Unilever, Nestlé and Malaysian Palm Oil Board. No other authors declare a conflict of interest.

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598

## FIGURE CAPTIONS

**Figure 1**: Study design for the controlled dietary intervention study (n=19). The study is conducted against the background of a standardized diet where incremental amounts of grapes were provided as an afternoon snack to the volunteers.

**Figure 2**: A) Urinary excretion kinetics of tartaric acid in 6 volunteers after consumption of 50g, 100g and 200g of grapes. The spectral region corresponding to the tartaric acid singlet at δ4.34 was absent in baseline samples (0h), visible in the 0-4h samples (red) and 4-8h samples (green) after grape intake, and subsequently decreased towards baseline levels in the 8-12h samples (blue) and 12-24h samples (magenta) after grape intake. B) 3D-PCA scores plot of spectra obtained from different varieties of red grapes with different geographic origins. Variance explained ($R^2_X$) for principal components (PC) 1, 2 and 3 is 30%, 13% and 11%, respectively. C) 3D-PCA scores plot of red grapes (red) vs green grapes (green) from different countries. $R^2_X$ for principal components (PC) 1, 2 and 3 is 25%, 13% and 11%, respectively. Key, origin: ☆Peru, □Namibia, ○Chile, ◊India, ▽South Africa; variety: Ralli, Magenta, Jack's Salute, Black Princess, Pink Muscat, Red Globe, Sharad, Flame, Crimson.

**Figure 3**: A) Kernel density estimate (KDE) of the predicted PLS-DA scores shows good separation between red (red cross, ×) and green (green circle, ○) grapes. An $R^2_Y$ of 0.65 shows the goodness of fit of the model and a $Q^2_Y$ of 0.52 shows good capability for prediction. B) PLS-DA scores plot. C) PLS-DA loadings plot. The $^1$H NMR peaks of 3 metabolites (phenylalanine, leucine and ethyl glucuronide) significantly different between red and green grapes are shown in individual panels, as

is tartaric acid for which no significant difference in the concentration was found between red and green grapes.

**Figure 4**: Calibration curves of tartaric acid (mMol) excreted in urine after the intake of 0, 50, 100 and 150g of grapes in A) the first spot urine samples 2h after grape intake, B) cumulative urine sample from after lunch to before dinner (CS2), C) cumulative urine sample from after dinner to next day fasting urine sample (CS3) and D) 24h urine samples. The shaded area represents the 95% confidence interval obtained using bootstrap resampling.

**Figure 5**: Bland-Altman plots of the actual and estimated grape intake in A) CS2 samples, B) CS3 samples and C) 24h urine samples, showing that the predictions of amount of grape intake in grams is close to the real intake of 50, 100 and 150g of grapes. The green line indicates mean ± 1 S.D., blue ± 2 S.D. and red ± 3 S.D.

599

**Figure 1**:

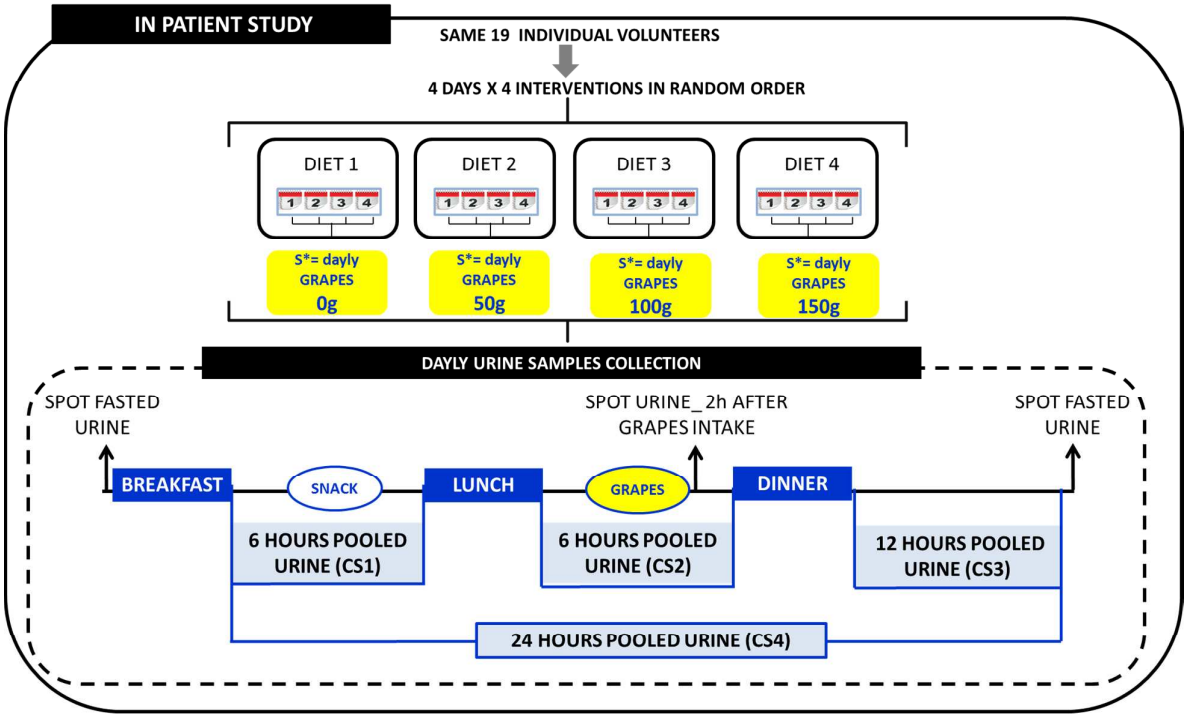

IN PATIENT STUDY
SAME 19 INDIVIDUAL VOLUNTEERS
4 DAYS X 4 INTERVENTIONS IN RANDOM ORDER
DIET 1
DIET 2
DIET 3
DIET 4
1 2 3 4
S*= dayly GRAPES 0g
S*= dayly GRAPES 50g
S*= dayly GRAPES 100g
S*= dayly GRAPES 150g
DAYLY URINE SAMPLES COLLECTION
SPOT FASTED URINE
SPOT URINE_ 2h AFTER GRAPES INTAKE
SPOT FASTED URINE
BREAKFAST
SNACK
LUNCH
GRAPES
DINNER
6 HOURS POOLED URINE (CS1)
6 HOURS POOLED URINE (CS2)
12 HOURS POOLED URINE (CS3)
24 HOURS POOLED URINE (CS4)

**Figure 2**:

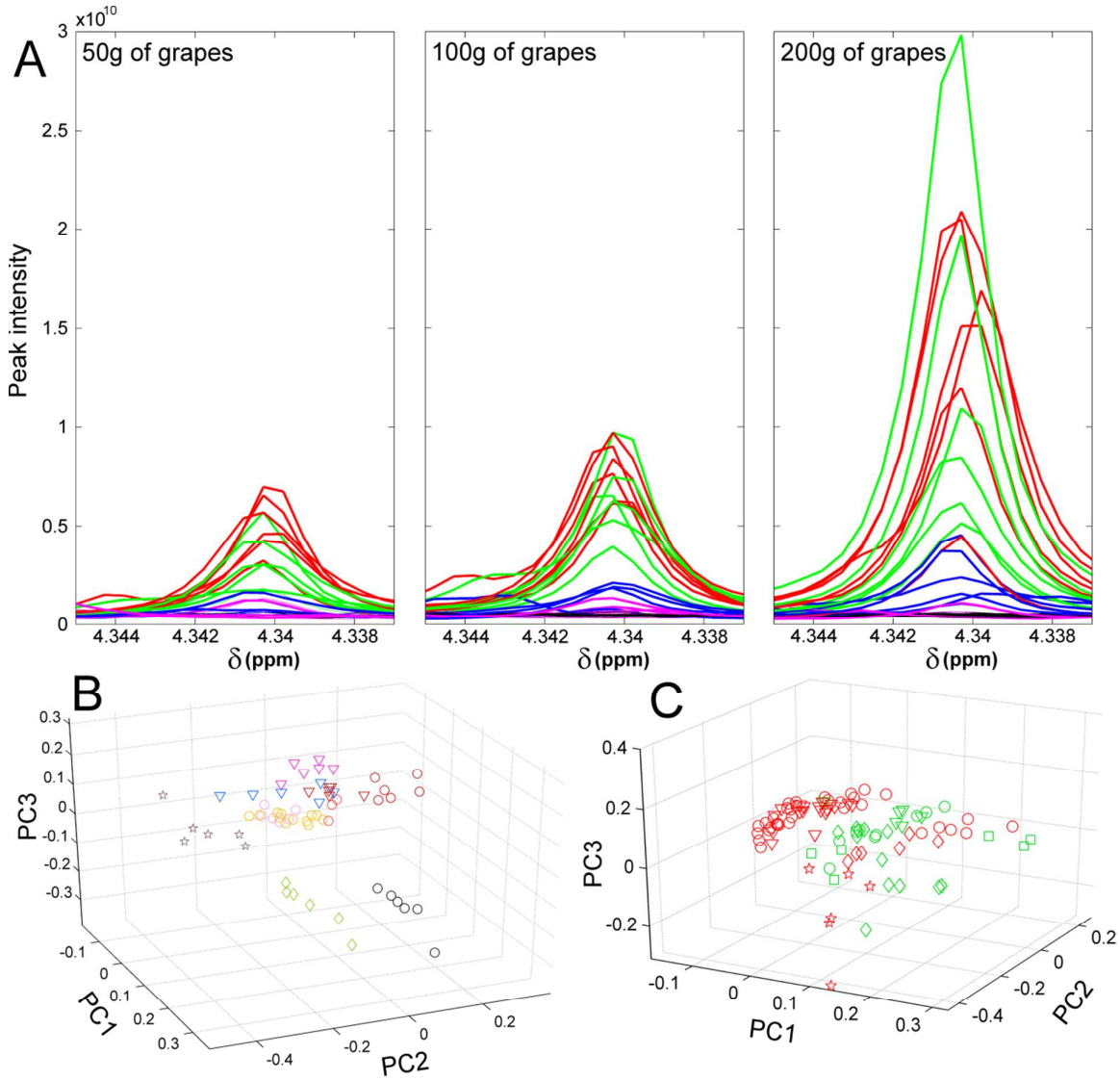

A
50g of grapes
100g of grapes
200g of grapes
x10^10
Peak intensity
δ (ppm)
B
C
PC1
PC2
PC3

**Figure 3:**

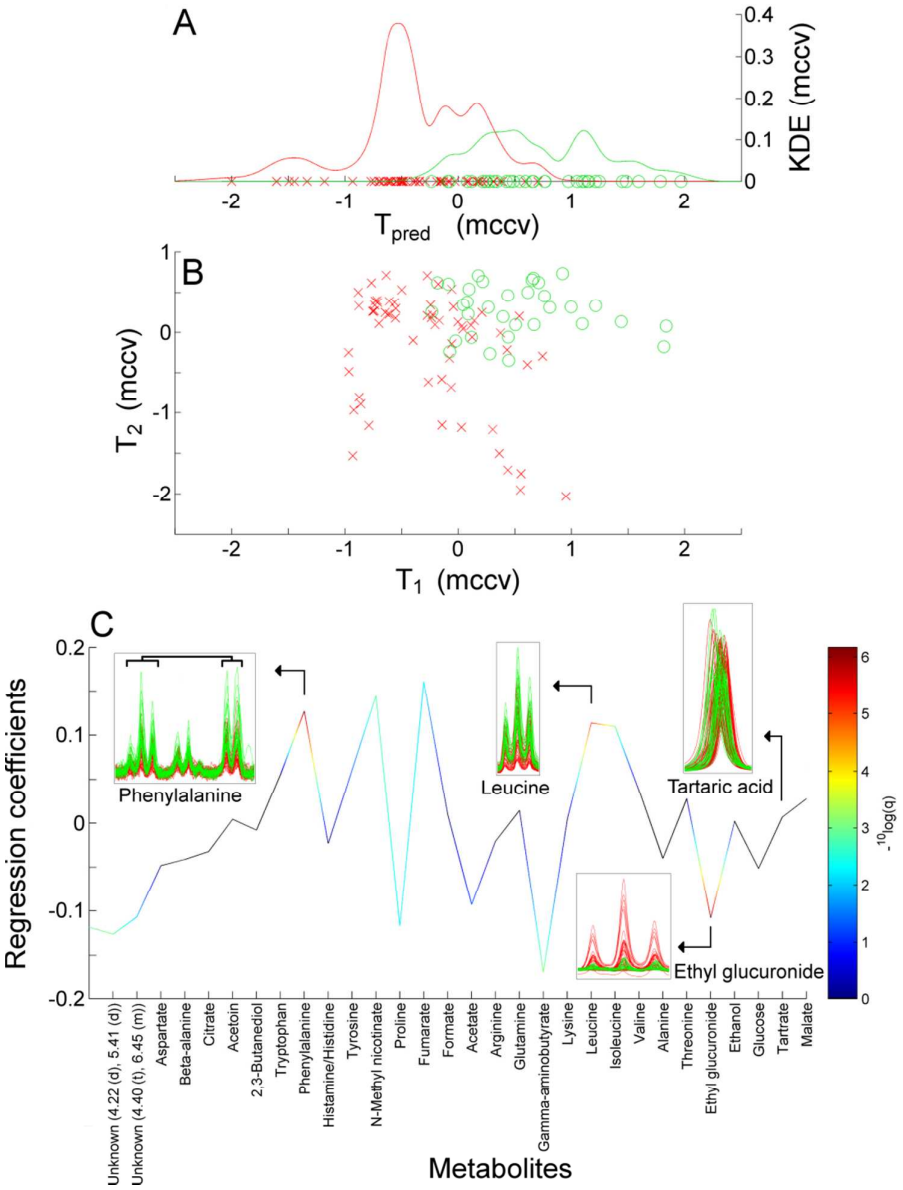

A
KDE (mccv)
$T_{pred}$ (mccv)
B
$T_2$ (mccv)
$T_1$ (mccv)
C
Regression coefficients
Phenylalanine
Leucine
Tartaric acid
Ethyl glucuronide
$-^{10}\log(q)$
Unknown (4.22 (d), 5.41 (d))
Unknown (4.40 (t), 6.45 (m))
Aspartate
Beta-alanine
Citrate
Acetoin
2,3-Butanediol
Tryptophan
Phenylalanine
Histamine/Histidine
Tyrosine
N-Methyl nicotinate
Proline
Fumarate
Formate
Acetate
Arginine
Glutamine
Gamma-aminobutyrate
Lysine
Leucine
Isoleucine
Valine
Alanine
Threonine
Ethyl glucuronide
Ethanol
Glucose
Tartrate
Malate
Metabolites

**Figure 4**:

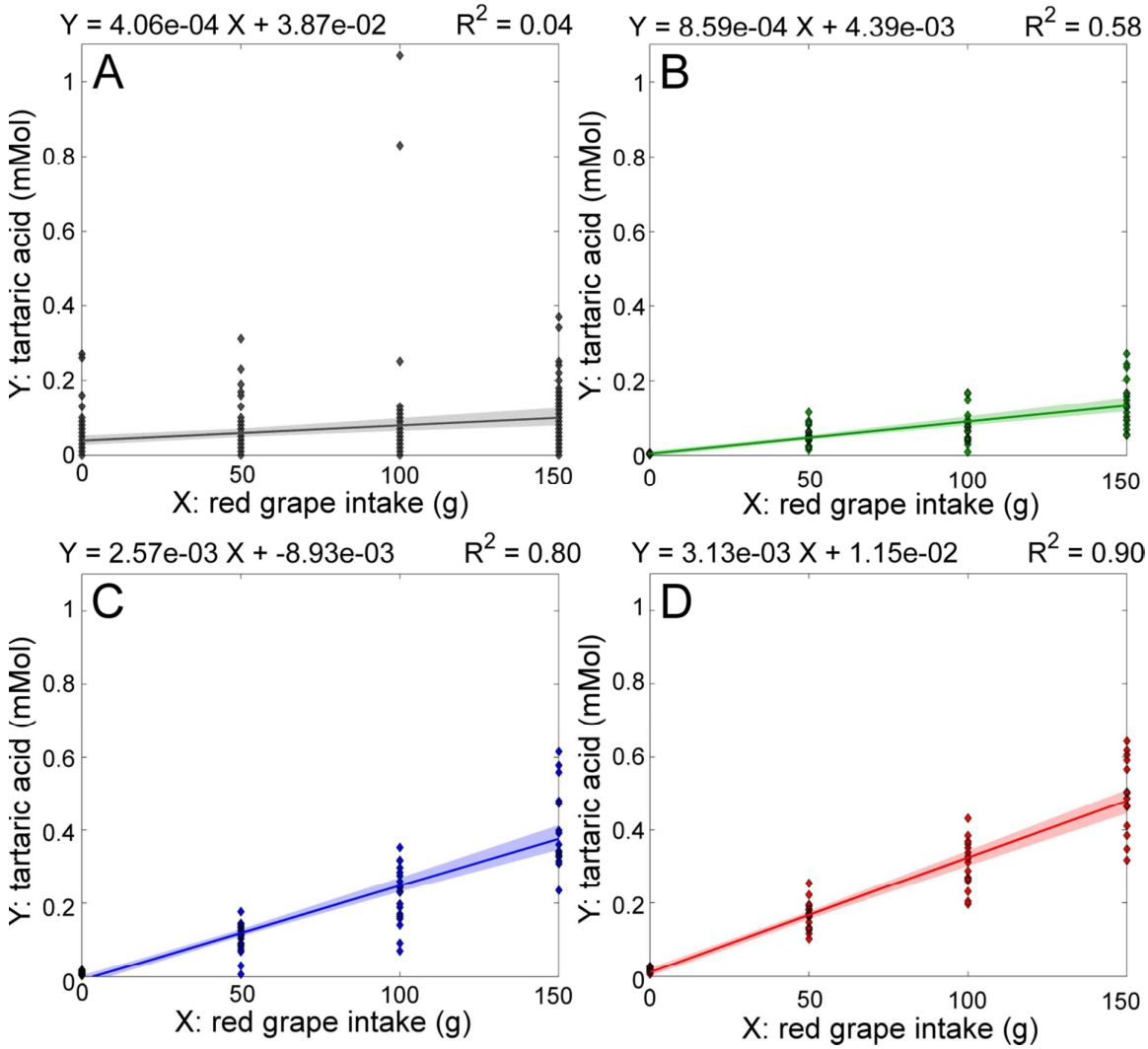
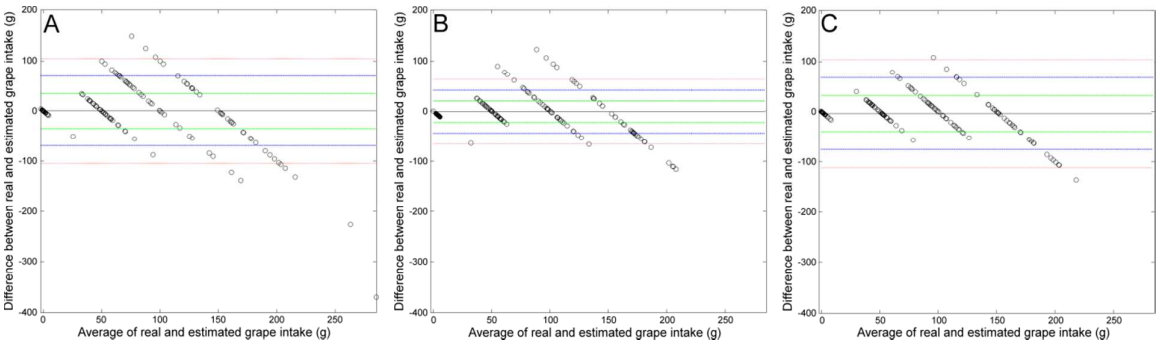
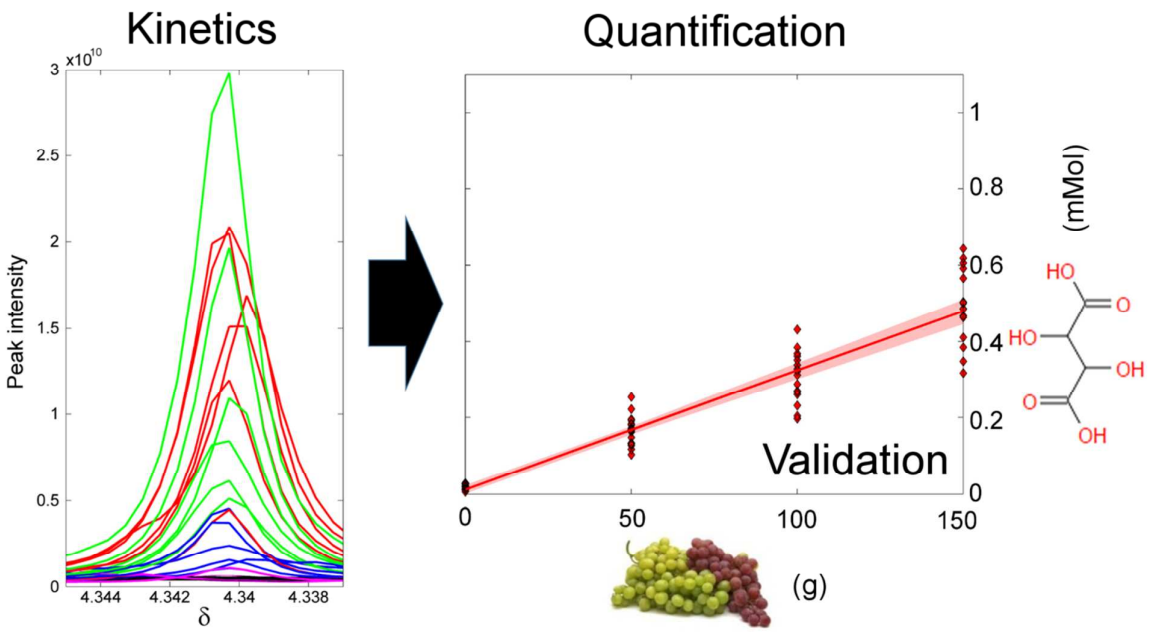

Y = 4.06e-04 X + 3.87e-02
R2 = 0.04
A
Y: tartaric acid (mMol)
X: red grape intake (g)
Y = 8.59e-04 X + 4.39e-03
R2 = 0.58
B
Y: tartaric acid (mMol)
X: red grape intake (g)
Y = 2.57e-03 X + -8.93e-03
R2 = 0.80
C
Y: tartaric acid (mMol)
X: red grape intake (g)
Y = 3.13e-03 X + 1.15e-02
R2 = 0.90
D
Y: tartaric acid (mMol)
X: red grape intake (g)

**Figure 5**:


A
B
C
Difference between real and estimated grape intake (g)
Average of real and estimated grape intake (g)
200
100
0
-100
-200
-300
-400
0
50
100
150
200
250

600

**TOC Graphic:**


Kinetics
Quantification
x10^10
Peak intensity
δ
(mMol)
Validation
(g)
HO
O
HO
OH
O
OH