"Raw food" diet: the effect of maximal temperature (46 ± 1 °C) on aflatoxin B_1 and oxalate contents in food

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Summary

"Raw food" diets are controversially accepted, but have been fashionable for a while and this trend seems to continue in the near future. Since the "raw food" diets are based on fruits, vegetables, nuts, seeds, sprouted grains and beans, higher daily intakes of this kind of food present a risk of higher intake of aflatoxins and oxalates, which consequently poses a health risk. The effect of the maximal temperature allowed at processing (46 ± 1 °C) has not yet been studied on aflatoxins and oxalates. In our study, their presence was determined by employing high pressure liquid chromatography with UV detection at the wavelength of 365 nm and 210 nm, respectively. A regression analysis was used to examine the changing of the content of oxalic acid and aflatoxin B_1 over time. The content of "naturally" present aflatoxin B_1 in selected dried fruits remained the same during 3 h of experiment at 46 ± 1 °C. On the contrary, the oxalate content in vegetables with low oxalic acid (tomatoes and chicory) decreased, whereas the temperature (46 ± 1 °C) had no effect on oxalic acid in spinach (higher oxalic content) and in pure solutions of oxalic acid in deionized water.

Keywords

"raw food" diet; temperature stability; dehydrated food; vegetables; aflatoxin B_1; oxalate

"Raw food" diets are nowadays controversially accepted. Although this kind of diets is not new and they have proponents all over the world, they became popular in the mid-19th century when Sylvester Graham suggested to eat uncooked foods to maintain health [1]. There is no exact definition of what "raw food" exactly is, the most used description is that this diet is an uncooked version of vegan diet.

It is not our intention to discuss *pro et contra* of the "raw food" diet. At this point, it might be important to quote the Academy of Nutrition and Dietetics (Chicago, Illinois, USA) and write their position statement: "The appropriately planned vegetarian, including vegan, diets are healthful, nutritionally adequate, and may provide health benefits in the prevention and treatment of certain diseases...." [2].

Since the "raw food" diets have been fashionable for a while [3] and this trend seems to continue in the near future, it was our task to examine the effect of the maximal processing temperature allowed (46 ± 1 °C) on two chemical groups of interests: aflatoxin and oxalate contents. These two chemical groups are important since "raw food" is based on fruits, vegetables, nuts, seeds, sprouted grains and beans. People, who mostly eat this kind of food have a higher probability of elevated intake of aflatoxins and oxalate in their bodies.

Ruthann Russo defined in his book [4] the term "uncooked" as the "raw food" which is heated to about 46 °C. The term "about" is used because "some say temperature cannot be higher than 110 °F (43 °C) while others claim 120 °F (49 °C) is the maximum. Clearly here is an area ripe for scientific testing" [4]. The temperature

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in "raw food" diet is needed mainly for dehydrating food and to increase the life span of the food. Besides, it helps to avoid boredom and increase the compliance of the lifestyle, by introducing the usage of seeds, nuts, etc. The "raw food" diet proponents claim that the content of nutrients remains almost unchanged up to this temperature and the enzymes are still active, which is believed to be helpful for digestion of foodstuff [5].

Dehydrated food (DF), e.g. dried fruits, vegetables, nuts and seeds, are natural sources of antioxidants, vitamins, minerals and oils and are therefore good substitutes of food of animal origin. Due to their long shelf-life, DF can provide a good alternative to fresh food. However, DF, especially nuts and dried fruits, are known to be possibly contaminated with mycotoxins [6, 7]. Aflatoxins are a group of "natural" mycotoxins, i.e. toxic and/or carcinogenic compounds produced by certain strains of *Aspergillus flavus, A. paraciticus* and *A. nomius*. The growth of fungal species and their production of aflatoxins in food and feedstuff are influenced by multiple variables, such as atmosphere, substrate, water activity, temperature, pH and time. Among all, the most critical are relative humidity and temperature in post-harvest processes (drying and storage) [5, 8–10].

The European Commission has set the maximum level for aflatoxin B_1 at 2–12 $\mu g \cdot kg^{-1}$, depending on the type of commodity [11]. Total aflatoxins value (expressed as sum of contents of aflatoxin B_1, B_2, G_1 and G_2) is specified in Codex Alimentarius Commission, which is set to 15 $\mu g \cdot kg^{-1}$ for food intended for further processing (peanuts, almonds, shelled Brazil nuts, hazelnuts and pistachios) [11].

The second group of substances, which might affect the health of humans on "raw food" diets, are oxalates. In fact, oxalates are salts, coming from oxalic acid, a basic constituent of vegetables and fruits. The excessive formation of a calcium oxalate stone and a consequent oxalate-induced renal failure [12] is a complex process due to several factors, including genetics and diet [13]. In general, "raw food" diet, which is based on the consumption of oxalate-rich fruit and vegetable juices obtained from juicing, might lead in some cases to severe health problems. Soaking and cooking of foodstuffs high in oxalate will reduce the oxalate content by leaching [14]. Vegetarians who follow the "raw food" diet have a higher intake of oxalates. The application of higher temperature may reduce the overall content of oxalates in different food matrices.

Thus, it was our aim to study the influence of temperature (46 ± 1 °C) on aflatoxin and oxalate contents upon exposition of 3 h (normal serving time). In the "raw food" diet, this temperature is the maximal temperature allowed to be kept for all steps, from food preparation to food consumption and it is limited by time, which is generally set to 3 h.

MATERIALS AND METHODS

Materials

Aflatoxin B_1 was purchased from Biopure (Cambridge, Massachusetts, USA). Oxalic acid was from Merck (Darmstadt, Germany). The other chemicals were all from Sigma Aldrich (St. Louis, Missouri, USA): sodium chloride, anhydrous sodium sulphate, solvents: methanol, acetonitrile and dichloromethane were all of analytical grade.

Sampling

Samples of nuts and dried fruits were bought in two shops and at the open market in Ljubljana, Slovenia in September, October and November 2016. All dried samples of fruits were immediately put in the freezer and kept at –18 °C till the analysis. Fresh samples were bought on the day of analysis. All samples were ground prior to the analysis and smaller portions of 10 g were weighed and stored at 5 °C in a refrigerator until used.

Analysis of aflatoxin B_1 in fruits and vegetables

Aflatoxin B_1 was extracted as described by Makun and co-authors with some modifications [15]. Fruits and vegetables were ground in a blender. Briefly, 10 g of each fresh or dried vegetables or fruits were mixed with 40 ml of 10% sodium chloride forming a heterogenous mixture. The mixtures were heated at 46 ± 1 °C in a water bath individually for 30, 60, 90 and 180 min in replicates. After heating, the heterogenous mixtures were filtered. A volume of 20 ml of filtrate was mixed with 50 ml of methanol-distilled water (85 : 15, v/v) and mixed for 15 min. Successively, 50 ml of dichloromethane were added to the mixture and shaken for additional 30 min. The mixture was poured into a separation funnel, the dichloromethane layer was separated and dried over anhydrous sodium sulphate. Dichloromethane was evaporated in a rotary evaporator and the dry residue was stored at 5 °C until analysis. Then, the residue was dissolved in 1 ml methanol, filtered through a cellulose-acetate membrane filter (Chromafil Xtra CA-45/25, pore size 0.45 μm; Macherey-Nagel, Düren, Germany) and injected into the high pressure liquid chromatography (HPLC) apparatus.

Aflatoxin B_1 was analysed on Agilent Technologies 1100 series HPLC system with UV detection (Agilent Technologies, Santa Clara, California, USA) at the wavelength of 365 nm. For the separation, an Ascentis Express C18 column (4.6 mm × 150 mm × 5 μm; Sigma-Aldrich) was used at the ambient temperature of 25 °C. The mobile phase was a mixture of 3 solvents (methanol, water and acetonitrile in a ratio of 50:40:10, v/v/v) and it was pumped through the HPLC system at a flow rate of 0.8 ml·min^{-1}. The injection volume was 10 μl. The calibration curve (1):

$$y = 56.40x - 0.12 \quad (1)$$

was linear in the concentration rage from 0.1 μg·ml^{-1} to 2.0 μg·ml^{-1}, with the correlation factor (R^2) of 0.999. The detection limit was 0.01 μg·ml^{-1}.

Analysis of oxalic acid in vegetables

The thermal stability of an analytical standard of oxalic acid was proven in water solutions before other experiments. No thermal decay was observed during 3 h (0, 30, 60, 90, 180 min) of experiment at 47 °C in pure solutions. In fact, the 4-point (20 mg·l^{-1}; 50 mg·l^{-1}; 80 mg·l^{-1}; 100 mg·l^{-1}) calibration curves (R^2 of 0.99 (0 min), 0.99 (30 min), 0.98 (60 min), 0.99 (90 min), 0.99 (180 min)) were statistically equal ($p > 0.05$). Fresh vegetables (50 g of each) were blended and mixed with 250 ml of distilled water and filtered. In order to avoid the delayed release of oxalic acid from the heterogenous matrix during the experiment, filtrates (25 ml) were heated at 46 ± 1 °C in a water bath for 0, 30, 60, 90 and 180 min. The samples were filtered through a cellulose-acetate membrane filter (Chromafil Xtra CA-45/25) and then injected into the HPLC system (Agilent Technologies 1100 series) with UV detection at the wavelength of 210 nm. For the separation, an Ascentis Express C18 column (4.6 mm × 150 mm × 5 μm) was used at the ambient temperature of 25 °C. The mobile phase was 25 mmol·l^{-1} sodium acetate, which was used at a flow rate of 1 ml·min^{-1}. The injection volume was 20 μl.

Statistical analysis

The experiments were done in duplicates. Regression analysis was used to examine the changing of the content of oxalic acid and aflatoxin B_1 over time. Simple regression analysis was carried out with the model:

$$Y_i = (b_0 + b_1 X_i) + \varepsilon_i \quad (2)$$

where Y_i is the outcome that we want to predict and X_i is the ith participant's score on the predictor variable. Here, b_1 is the gradient of the straight line fitted to the data and b_0 is the intercept of that line. These parameters, b_1 and b_0, are known as the regression coefficents. Residual term, ε_i, represents the difference between the score predicted by the line for participant i and the score that participant i actually obtained.

The overall fitting of the model was tested by the analysis of variance (ANOVA) with SPSS Statistics software (IBM, Armonk, New York, USA). The critical value for testing the effect of process time on the content of aflatoxin B_1 (at the level of significance of 0.05) was $F(1, 8) = 5.32$, and for oxalic acid $F(1, 3) = 10.1$.

RESULTS AND DISCUSSION

The effect of temperature on aflatoxin B_1

Cashew nuts, dried plums and a mixture of nuts with dried fruits were chosen as model substances for the experiment. Those ingredients are usually used in well known "raw cakes" and are consumed by individuals keeping to the "raw food" diet in greater amounts then the usual daily intake. The elevated content of aflatoxin B_1 on/in dried fruits and nuts may represent a significant health hazard for people on "raw food" diets. According to our knowledge, there no specific report has been published on the effect of the maximal temperature of 46 ± 1 °C (for a maximum of 3 h, according to the "best serving practice") on aflatoxin B_1 contents, in order to give explicite answers to "raw foodists". In the previously cited literature, it is stated that higher water content and elevated temperatures may lead to extensive aflatoxin B_1 production.

According to Gallo et al. [16], involving nuts as a substrate, the maximal fungal growth and aflatoxin production were observed at 28 °C and at a water activity a_w of 0.96 on an almond medium. At the same time, at 20 °C and below a_w of 0.93, no fungal growth was noticed. On the other hand, at 37 °C good fungal growth but a very low aflatoxin B_1 production occurred at a_w of 0.93–0.99 [16]. In a study of Hanif et al. [17], regarding the drying process, the maximal aflatoxins (aflatoxin B_1 and aflatoxin G_1) production, 31.7 μg·kg^{-1}, was recorded in persimmons treated with aloe vera and dried under a temperature of 40 °C, while the minimum of 7.1 μg·kg^{-1} was recorded in persimmons treated with honey and dried at a temperature of 60 °C [17]. Similarly, dehydration at 40 °C and 50 °C was considered as representing a microbial hazard in case of buckwheat-based "raw food" [10]. It could be concluded that the increase in temperature of the drying system decrease, in general, the con-

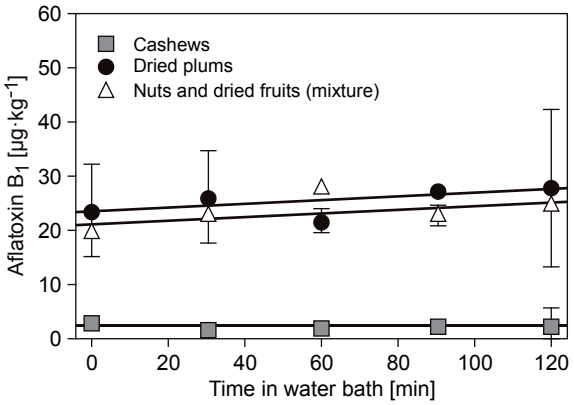

Fig. 1. The content of aflatoxin B_1 during the experiment at 46 ± 1 °C.

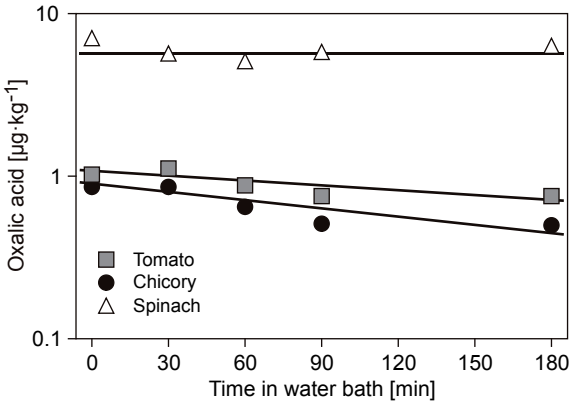

Fig. 2. The content of oxalic acid in vegetables during the experiment at 46 ± 1 °C.

tamination by fungi. However, in case of "raw food", elevated temperatures are required to be avoided since the temperature is the critical point of the diet.

Since the ideal temperature for aflatoxins production is 28–30 °C, together with higher water activity values of substrates [18], it was our task to study what is going on at the maximal allowed temperature for "raw food" diet. As reported by Abdel-Hadi et al. [19], the composition of substrate can affect the aflatoxin production by *A. flavus*. By varying the combination of the parameters involved in aflatoxin biosynthesis, toxin production can be completely inhibited or fully activated. It is therefore fundamental to know which combinations can control, or be conducive, to aflatoxin production in crops or substrates [19].

Our aim was to follow the kinetics of degradation or to monitor the constancy of aflatoxin B_1 during 3 h of food exposure to 46 ± 1 °C. In fact, the amount of aflatoxin B_1 present on/in the substrate changed only slightly over time. The linear regression was used to prove the constant content of aflatoxin B_1 on/in different substrates during 3 h of experiment. If the content of the analysed component (aflatoxin B_1) during the experiment remained the same, the slope of the regression line (b_1) was not significantly different from 0 (Fig. 1). The effect of time on the content of aflatoxin B_1 was not confirmed for any of the studied food (Tab. 1).

Very low values of R^2 tell us that time itself can account from 5 % to 24 % of the variation in the content of aflatoxin B_1 in analysed food. In other words, the line representing the relationship between the content of aflatoxin B_1 and time is flat ($b_1 \approx 0$; $p > 0.05$), which means that, as the time changes, the content of aflatoxin B_1 does not change.

Tab. 1. Parameters describing the effect of processing time on the content of aflatoxin B_1.

Type of food	R^2	F	Significance
Cashew nuts	0.06	0.047	0.835
Dried plums	0.05	0.412	0.539
Nuts and dried fruits (mixture)	0.24	2.574	0.147

R^2 – the proportion af the total variation in Y that is explained by the fitted regression.
F – F-ratio, a measure of the ratio of the variation explained by the model and the variation explained by the unsystematic factor.

Tab. 2. Calibration curves of oxalic acid according to different immersion times in waterbath at 46 ± 1 °C.

Time at 46 ± 1 °C [min]	R^2	Linear curves
0	0.99	$y = 0.65x + 18.6$
30	0.99	$y = 0.64x + 15.1$
60	0.99	$y = 0.57x + 23.7$
90	0.99	$y = 0.52x + 23.3$
180	0.99	$y = 0.67x + 16.3$

The effect of temperature on oxalic acid

Many experimental and several theoretical studies were previously undertaken on thermal decomposition of oxalic acid, which established that the major decomposition products of oxalic acid are CO_2, HCOOH, CO and H_2O, over the temperature range of 400–430 K (127–157 °C) [20]. Decomposition of oxalate species follows a first-order rate law. Degradation rate increases with decreasing pH [21].

The only experiment regarding oxalic acid at lower temperatures (17–60 °C) dealt with the solu-

Tab. 3. Parameters describing the effect of processing time on the content of oxalic acid.

Samples of vegetables	R^2	F	Significance	Linear curves
Tomato	0.70	6.895	0.079	$y = 2.01 - 0{,}001x$
Chicory	0.75	8.831	0.059	$y = 1.74 - 0{,}002x$
Spinach	0.01	0.042	0.850	$y = 2.66 - 0{,}00007x$

R^2 – the proportion af the total variation in Y that is explained by the fitted regression.
F – F-ratio, a measure of the ratio of the variation explained by the model and the variation explained by the unsystematic factor.

bility of oxalic acid in different solvents like water, chloroform, acetone, alcohol and many percentile solutions of different solvents at different temperatures (17, 25, 33, 39, 45 and 60 °C) which was done by HUSSAIN et al. [22]. Since the research dealt with solubility, behind lied the statement of thermal stability at those temperatures.

In order to compare the oxalic acid content in different food matrices and the effect of temperature, it was necessary to prove the thermal stability of pure solutions of oxalic acid in deionized water. In our experiments, we proved the thermal stability of oxalic acid solutions during 3 h of immersion in water bath at 46 ± 1 °C (Tab. 2). Analysis of covariance (ANCOVA) was used to test the homogenity of the regression coefficient (Tab. 2). The analysis showed no significant differences among regression slopes ($F = 0.416$; $p = 0.744$), which confirmed our assumption that the relationship between the oxalic acid standard solutions of different concentrations immersed in the water bath and the time was the same in all cases.

In contrast, a different pattern of oxalic acid was determined in real fresh samples of tomato and chicory. Although the results showed that the content of oxalic acid in samples decreased over time (Fig. 2), we were not able to confirm this feature statistically (Tab. 3). However, the values of significance were approaching to the threshold of 5 %, which may nevertheless indicate the reduction in content, which might be confirmed with further studies.

Relatively high values of R^2 in case of tomato and chicory tell us that time itself can account for 70–75 % of variation in the content of oxalic acid in the analysed food. The decline of content of oxalic acid in tomato and chicory was especially visible in the first 90 min of the experiment. After that time, the reduction stopped and reached a plateau, while the content of oxalic acid in spinach remained more or less the same throughout the experiment.

The reduction of oxalic acid and, consequently, the decreased amount of calcium oxalate in formed in the human body, may help to prevent or decrease the formation of kidney stones, as well as development of other renal disorders escpecially in those humans who are predominantly on "raw food" diets.

CONCLUSIONS

The exposure to aflatoxin B_1 and high intake of oxalic acid represent a serious health hazard for people on "raw food" diets. The content of "naturally" present aflatoxin B_1 remained the same during 3 h of experiment at 46 ± 1 °C. Interesting results gave the effect of temperature on the oxalate content in vegetables. At a constant temperature of 46 ± 1 °C, the content of oxalate in some vegetables with low oxalic acid content (tomatoes and chicory) decreased, whereas no such effect on oxalic acid was determined in spinach (vegetable with a higher oxalic acid content).

Acknowledgements

The authors acknowledge the financial support from the Slovenian Research Agency (research core funding No. P3-0388, Mechanisms of health protection (Mehanizmi varovanja zdravja)).

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Received 29 May 2017; 1st revised 5 July 2017; accepted 17 July 2017; published online 14 September 2017.