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Content of *trans*-fatty acid isomers in bakery products on the Slovenian market

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Abstract

The objective of the present study was to provide data on the content of different isomers of *trans*-fatty acids (TFAs) in bakery products available on the Slovenian market. The samples were categorised as 'bread' (n = 16), 'cookies/biscuits/wafers' (76), 'dough snacks' (21) and 'dough' (4); of these, 71 were prepacked, and 46 were non-prepacked. These investigated foods contained (% total FAs) up to 31% TFAs, with *trans*-C18:1 as 29.7% and *trans*-C18:2 as 1.3%. In the 'cookies/biscuits/wafers' category, the main *trans*-C18:1 isomers were *t*9-C18:1 and *t*10-C18:1, while in 'dough snacks', *t*11-C18:1 was the highest. Among the *trans*-C18:2 isomers, *t/c*-C18:2 and *c/t*-C18:2 were in equal amounts, while *t/t*-C18:2 was present mainly in the samples with higher TFA content. Using food consumption data we calculated daily consumed TFAs for different population groups and showed that among investigated food categories 'cookies/biscuits/wafers' were the most important source of TFAs. Bakery products could be categorized according to indices of atherogenicity and thrombogenicity, as well as TFA and *trans*-C18:2 contents.

Keywords: bakery products, *trans*-fatty acid isomers, atherogenicity, thrombogenicity, linear discriminant analysis

1. Introduction

Two sources of *trans*-fatty acids (TFAs) are known in the human diet: those naturally present in meat and in the milk of ruminants produced through biohydrogenation by their microflora in rumen; and those produced industrially. These two types differ considerably in their isomer distributions, which are linked to the position of the *trans* double bond along the carbon chain. The main FA in industrially produced TFA is elaidic acid (*t*9-C18:1), followed by *t*10-C18:1, whereas ruminants principally produce vaccenic acid (*t*11-C18:1), which represents up to 50% of all of their TFAs (excluding conjugated FAs) (Bainbridge, Cersosimo, Wright & Kraft, 2016). The factors that affect the TFA composition of bovine milk include lactation stage and breed (Bainbridge et al., 2016), although animal diet is regarded as the key factor (Ferlay, Bernard, Meynadier & Malpuech-Brugère, 2017; Alothman et al., 2019). A ruminant diet based on grass results in a higher total TFA content, as well as a higher ratio of the dominating *t*11-C18:1, which might shift to *t*10-C18:1 if the diet is rich in concentrate or in digestible carbohydrates (Ferlay et al., 2017). Partially hydrogenated vegetable oils (PHVOs) are the main source of industrial TFAs in the human diet, and various regulatory approaches are used across the world to separate these from the food supply (Ghebreyesus & Frieden, 2018; Pravst, 2015; WHO, 2018).

Mono-unsaturated TFAs are the most common, but various di-unsaturated *cis-trans*, *trans-cis* and *trans-trans* isomers also occur. These double bonds can be at different positions along the chain, with *trans* isomers from *t*4 to *t*16 known.

Trans-fatty acids are known to affect human health, with the evidence strongest for the association of the intake of TFAs and the risks of some non-communicable diseases, particularly cardiovascular disease (CVD) (Chowdhury, Johnson & Steur, 2014; De Souza et al., 2015; Brandt, Myerson, Perraillon & Polonsky, 2017) and diabetes (Islam, Amin, Siddiqui,

Hossain, Sultana & Kabir, 2019). Although the mechanisms behind these effects are still not completely understood, various studies have indicated pro-inflammatory activities for TFAs (Hadj Ahmed et al., 2018; Baer, Judd, Clevidence & Tracy, 2004; Mozaffarian et al., 2004), with the consumption of TFAs associated with higher levels of inflammatory markers (Valenzuela, Baker, Miles & Calder, 2019). A positive association between dietary inflammatory index and plasma TFAs was reported in a National Health and Nutrition Examination Survey (NHANES) study conducted on adults in the USA (Mazidi, Gao, Shivappa, Wirth, Hebert & Kengne, 2017). While TFAs still remain a problem in our diet, a further study on adults in the USA showed reductions in plasma TFA concentrations from 1999-2000 to 2009-2010, due to the reduction of TFAs in the food supply (Vesper et al., 2017).

When comparing PHVOs rich in *t*9-C18:1 acid with ruminant fat rich in *t*11-C18:1 in human hepatocyte cells, these two TFAs might well elicit different responses. Elaidic acid might have a greater impact on cholesterol synthesis through increasing the proteins involved in the process, in comparison to *t*11-C18:1 (Krogager et al., 2015; Vendel Nielsen et al., 2013). However, according to Stender (2015), both ruminant and PHVO TFAs have similar effects on LDL cholesterol, while the ruminant TFAs are easier to remove from foods.

Koba et al. (2019) reported similar plasma contents of total TFAs in healthy Japanese men (control) and in patients with acute coronary syndrome, where the patients had been diagnosed with lower levels of palmitelaidic acid (*t*7-C16:1), the *trans*-isomer of palmitoleic acid, which is a ruminant-derived TFA. However, the acute coronary syndrome group had lower HDL cholesterol and significantly higher industrially produced total *trans*-C18:2 and total *trans*-C18:1 isomers, in comparison to the control group. A study by Liu et al. (2019) reported significant positive association of plasma *t*9-C18:1 acid with symptoms of depression in adults. They also reported a positive, although not significant, association between symptoms of depression and total TFAs, linolelaidic acid, palmitelaidic acid and *t*11-C18:1.

Some countries limit the TFA content in foods to 2% of total FAs, while some restrict the use of PHVOs (Ghebreyesus & Frieden, 2018; Pravst, 2015). However, in countries where regulatory limits have not yet been implemented, PHVOs can still be used to manufacture a variety of food products. Costa, Cruz, Graça, Breda & Casal (2016) reported the highest contents of TFAs in biscuits, wafers and cookies, while Vučić, Arsić, Petrović, Milanović, Gurinović, & Glibetić (2015) reported modest quantities of TFAs in margarines. On the other hand, in our previous investigation (Abramovič et al., 2018) only two of the 43 analysed margarine samples in Slovenia contained more than 2% TFAs, in terms of the total FAs, with higher TFA contents in many shortenings. Thus, we reported mainly *t*9-C18:1 in all of the shortening samples, followed by *t*10-C18:1, (*t*6-*t*8)-C18:1 and *t*12-C18:1. Similar distributions of TFAs were also shown in margarines, with the exception of one sample, where the main isomer was *t*10-C18:1.

The aim of the present study was to quantify the total TFAs and the single *trans*-C18:1 and *trans*-C18:2 isomers in different categories of bakery products (bread, cookies/biscuits/wafers, dough snacks, dough) from the Slovenian food supply. Also, the indices of atherogenicity (AI) and thrombogenicity (TI) were calculated and are related to estimations of their effects on human health.

2. Materials and methods

2.1. Sample collection

The total number of samples analysed was 117, which comprised: bread, 16; cookies/biscuits/wafers, 76; dough snacks, 21; dough, 4. The foods were purchased in randomly selected Slovenian supermarkets in 2016. We sampled products of various brands which were available on shelves of the selected stores at the time of purchase/ sampling.

120 The sampling approach was as previously described by Kušar et al. (2021). On the basis
121 that Zupanič, Hribar, Pivk- Kupirovič, Kušar, Žmitek & Pravst (2018) showed significant use
122 of PHVOs in some categories of prepacked foods, and especially in bakery products, we
123 sampled a limited number of food products that were labelled to contain PHVOs, and also some
124 foods that appeared to be produced without the use of PHVOs. We also included specific types
125 of non-prepacked foods (e.g., *Burek,* croissant) that are known from previous studies to often
126 contain notable amounts of TFAs. No labelling is required for these food products, so we could
127 not stratify the sampling of such non-prepacked foods for the use of PHVOs.
128 The majority of samples were prepacked (n = 71), while the others were non-prepacked
129 (n = 46). The samples were delivered to the laboratory and were milled using a laboratory mill
130 (Retsch GRINDOMIX GM 200, Germany) and stored at -18 °C in vacuum bags until the
131 analyses were performed.
132
133 ***2.2. Preparation of fatty acid methyl esters***
134 Before extraction and methylation of the homogenised samples, 200 µL heptadecanoic acid
135 (Merck, Darmstadt, Germany) was added as internal standard. The lipids were extracted from
136 these samples with dichloromethane. Sodium hydroxide in methanol was added to the samples
137 in a test-tube, with heating at 90 °C for 50 min in a water bath. After cooling, boron trifluoride
138 in methanol was added, followed by 10% NaCl. Hexane was used as the solvent for the fatty
139 acid methyl esters (FAMEs), with the samples transferred to a sample bottle containing
140 anhydrous sodium sulphate. The FAMEs were extracted by vortexing for 1 min. After
141 centrifugation (1500 rpm, 5 min, 25 °C), the hexane layer was transferred into vials, which were
142 sealed and stored at -20 °C until the chromatographic analyses. The TFA levels were
143 determined as three replicates, using gas-liquid chromatography.
144

2.3. Gas chromatography analysis

The FA composition, total TFAs and specific TFA isomers of the samples were determined by gas-liquid chromatography with *in-situ trans*-esterification (Park & Goins, 1994). The FAs were identified as previously described by Abramovič et al. (2018).

The FAMEs were analysed using two different gas-liquid chromatography methods. In the first, 1-μL samples were injected into the gas chromatograph (6890 GC; Agilent Technologies) with a flame ionisation detector and a capillary column (BPX-70; 120 m × 250 μm × 0.25 μm). Separation and detection of the FAMEs were performed under the following conditions: oven programme, 130 °C (0 min); 1 °C/min to 220 °C (hold 10 min). The injector (split:splitless, 1:50) and detector temperatures were 240 °C and 280 °C, respectively. Helium was used as the carrier gas (24 cm/s). The second method for the FAMEs used a gas chromatograph (Thermo Trace GC) equipped with a flame ionisation detector and a capillary column (SP2560; 100 m × 250 μm × 0.25 μm). Separation and detection of the FAMEs here were performed using the oven temperature programme: 120 °C (1 min); 20 °C/min to 175 °C (hold 30 min); 5 °C/min to 220 °C, (hold for 10 min). The injector and detector temperatures were maintained at 250 °C. The injection of 1-μL samples used a split ratio of 1:100. The carrier gas was hydrogen (1.5 mL/min, constant flow).

2.3.1. Identification and quantification of fatty acid methyl esters

The identification of the FAMEs after the GC analysis was accomplished by comparing the retention times of the peaks in the sample with those of commercial standards (Supelco FAME mix of 37 components; Cat. No. 18919-1AMP). The standard mixture was also used for determination of the response factor (Rfi) for each FA. Quantification of each of the FAs was achieved using the peak areas of the internal standards (C17:0 - heptadecanoic acid). Each FA was calculated and is expressed as the percentage of the total FAs (g/100 g).

Additional identification of the *cis*- and *trans*-C18:1 isomers was performed by means of thin layer chromatography (Buchgraber & Ulberth, 2001) with Ag^+ as described by Abramovič et al. (2018).

2.4. Calculation of daily consumed TFAs for different population groups (infants, adolescents, adults, elderly) in Slovenia

The daily consumed TFAs were calculated for different groups of the Slovenian population (infants, adolescents, adults, elderly) and expressed in g of TFAs per day (g/day) for each selected food category. These assessments were based on the daily amount of food consumption data from the EFSA Comprehensive European Food Consumption Database. (2021) and the mean and maximal TFAs contents that were determined in different food categories.

2.5. Calculation atherogenicity and thrombogenicity indices

The AI indicates the ratio between the sum of the main pro-atherogenic FAs (i.e., TFAs, saturated FAs [SFAs] with chain length 12, 14 or 16 C atoms) and the main classes of anti-atherogenic unsaturated FAs (MUFAs; *n*-6 FAs, *n*-3 FAs). The TI is related to the tendency to form clots in blood vessels. This is defined as the ratio between the pro-thrombogenetic FAs (TFAs, stearic acid [C18:0], myristic acid [C14:0], palmitic acid [C16:0]) and the anti-thrombogenetic FAs (MUFAs, *n*-3 FAs, *n*-6 FAs).

The AI and TI were calculated according to Equations (1) and (2), respectively, as proposed by Ulbricht & Southgate (1991) and modified by Vučić et al. (2015).

$$AI = (C12{:}0 + 4 \times [C14{:}0] + C16{:}0 + TFA) / (MUFA + n\text{-}6 + n\text{-}3) \quad (1);$$

194 $TI = (C14:0 + C16:0 + C18:0 + TFA) / (0.5 \times MUFA + 0.5 \times [n\text{-}6] + 3 \times [n\text{-}$

195 $3] + [n\text{-}3 / n\text{-}6])$ (2).

196

197 All of the FA contents are reported as % total FA. TFAs were added into the totals of

198 the individual SFAs because they have similar atherogenic properties as the SFAs. As myristic

199 acid is considered to be 4-fold more atherogenic than the other SFAs, it was assigned a

200 coefficient of 4. As the polyunsaturated *n*-6 FAs and MUFAs are less antithrombogenic than

201 the *n*-3 FAs, they were assigned a coefficient of 0.5. The *n*-3 FAs were assigned a coefficient

202 of 3 (Pikul, Wójtowski, Danków, Kuczyńska & Łojek, 2008).

203

204 ***2.6. Statistical analysis***

205 Statistical analysis was performed using SPSS version 22 for Windows, as the evaluation

206 version (SPSS Inc., Chicago, IL, USA). All of the data were tested for normal distributions.

207 Mean and standard deviations of triplicate determinations are reported. One-way ANOVA was

208 carried out to a 95% confidence level ($P \leq 0.05$). Multivariate analysis included factor analysis

209 and linear discriminant analysis (LDA).

210

211 **3. Results and discussion**

212

213 ***3.1. Fatty acid profile***

214 There was considerable variability of the contents of SFAs, MUFAs, polyunsaturated FAs

215 (PUFAs), *n*-6 FAs, *n*-3 FAs and TFAs between and within the investigated food categories

216 (Figure 1). The results show the high variability in the composition of these bakery products in

217 the food supply. It thus appeared that the producers of these bakery products had used a wide

218 range of available shortenings or fats.

In general for these products, the UFAs predominated over the SFAs, and the *n*-6 FAs were at much higher levels than the *n*-3 FAs (Figure 1). In the category of 'bread', the mean PUFA content exceeded the mean MUFA content. Among SFAs the highest proportion of C16:0 was seen in 15 samples of 'bread', although in one sample (breadstick), more C12:0 (21.3%) and C14:0 (8.2%) were seen. Both of these medium chain FAs are probably due to the presence of coconut oil, which was also declared on the label of this product. Vegetable fats are rich in C12:0 and C14:0 FAs, especially coconut and palm kernel oils (Santos, Cruz & Casal, 2015).

In the 'cookies/biscuits/wafers' category, the mean MUFA content exceeded that of the PUFAs. Oleic acid (C18:1*n*-9) was the main MUFA (29.9%), while the most prevalent PUFA was linoleic acid (C18:2*n*-6), at 13.6%. In almost all of the samples of this category, the SFAs were mainly composed of C16:0, followed by short and medium chain FAs. Only four samples showed mainly C12:0, followed by C14:0.

In the 'dough snacks' category, the SFAs were mainly composed of C16:0. Oleic acid represented 27.8% of the MUFAs. For the PUFA profile, C18:2*n*-6 predominated in all of the samples (mean ±standard deviation, 30.2% ±9.4%), while the *n*-3 FAs were found in very small amounts.

In the 'dough' category, the mean SFA content slightly exceeded that of the UFAs. The main SFA was C16:0, at 46.2% (±2.9%) of the total FAs. The MUFAs were the major UFA group. The analysed 'dough' samples were also shown here to be good sources of C18:1*n*-9 (34.3% ±2.6%). The most prevalent PUFAs in 'dough' samples were *n*-6 FAs, as particularly C18:2*n*-6 (10.7%).

The most abundant FAs in the categories of 'bread' and 'dough snacks' was C18:2*n*-6, while C16:0 dominated in the 'cookies/biscuits/wafers' and 'dough' categories. The most abundant MUFA in all of these food categories was C18:1*n*-9 (21.5%–34.3%), which might

have originated from palm or rapeseed oil. On the other hand, C18:1*n*-9 might also originate from the partial hydrogenation of C18:2*n*-6, when accompanied by increased contents of TFAs. Oleic acid is considered to be beneficial for health due to its antiatherogenic effects. Its increased consumption can improve the *n*-6/*n*-3 ratio by displacing the oils that contribute to the *n*-6 FA intake (Simopoulos, 2008). All of the samples analysed in our study had relatively high *n*-6/*n*-3 ratios (from 25 in 'bread', to 61 in 'dough snacks'). The optimal *n*-6/*n*-3 ratio is from 1 to 4 (Simopoulos, 2008). As found in many modern-day diets, a high *n*-6/*n*-3 ratio can promote pathogenesis of many diseases, including thrombosis, inflammatory diseases and CVD (Wijendran & Hayes, 2004).

It is important to consider which type of FAs should be used to replace industrial TFAs in the reformulation of food products. From a technological point of view, SFAs are the first choice, due to their similar texture, taste and shelf life, compared to TFAs. However, inter-esterified formulations of fat (where TFAs are replaced by MUFAs and PUFAs) offer a healthier alternative. National health organisations should advise the food industry, to support more efficient food reformulation towards healthier foods. In line with this, the REPLACE action plan was launched by the World Health Organisation (WHO), with the goal of eliminating industrially produced TFAs from the global food supply by 2023 (WHO, 2018). One of the key objectives in this plan is that food manufacturers and restaurants should choose to use alternative fats in food production, to maximise health benefits. The alternatives here should be low in TFAs and SFAs. Governments should also support consumers and educate them on how to minimise the intake of both TFAs and SFAs. These steps will help to minimise the public health burdens of CVD and other diseases.

3.2. Trans-fatty acid content

There was a high variation in the TFA contents within and between all of these categories of bakery products, except for 'dough' (Figure 1). The mean total TFA content of all of the analysed samples independent of the food category (n = 117) was 2.0% ± 2.3% of total FAs; however, the median was 1.1%. The mean TFA content of each category except 'cookies/biscuits/wafers' was <2%. There were higher amounts of TFAs in 'cookies/biscuits/wafers', followed by 'dough snacks'. In approximately 34% of all of these analysed samples, the TFA content was <2%. This shows that there are significant amounts of TFAs in quite a lot of 'cookies/biscuits/wafers' and 'dough snacks', rather than in 'bread' and 'dough'.

Some EU Member States are already taking actions at a national level, but harmonised regulatory limitations on TFA contents in food products in the European Union (EU) would be the more efficient way to ensure wide protection for the entire EU population, even in countries where data on TFA contents of food products are not available. While the regulatory limit of 2 g TFAs per 100 g total FAs will be enforced in the EU from 1 April, 2021 (EC, 2019), this limit was already introduced in Slovenia in 2019 (Uradni list, 2018). On the other hand, at the time of sampling for the present study, the TFA content in foods was not legally restricted, although qualitative information about the presence of PHVOs needed to be disclosed on labels of prepacked foods (EC, 2011).

In the present study, >2% TFAs was found in 19 of the 46 non-prepacked food samples. On this basis, with food labelling only required for prepacked food products, this suggests that a regulatory TFA limit is very relevant for both prepacked and non-prepacked foods. Of note here, within the 21 prepacked samples with >2% TFAs, more than half of these were produced in the Balkan countries.

Previous studies have shown that an intake of about 5 g TFAs/day is associated with a 25% increased risk of coronary heart disease (Mozaffarian, Katan, Ascherio, Stampfer &

293 Willett, 2006). Some public health organisations have therefore recommended that TFA intake
294 should be reduced as much as possible (Stender, Astrup & Dyerberg, 2016). The major sources
295 of industrially produced TFAs in the food supply are bakery products, deep-fried foods, pre-
296 packed snack foods, margarines, shortenings and crackers (Mozaffarian et al., 2006).
297 Many studies have also reported increased risk for CVD with increased levels of *trans*-
298 C18:2 isomers, and particularly for *t/t*-C18:2, which showed a higher risk than the *trans*-C18:1
299 isomers. Among the various types of foods that contain PHVOs, bakery products were the only
300 dietary source that showed significant association with plasma biomarkers for *trans*-C18:2
301 (Chowdhury et al., 2014). However, previous studies have shown that dietary sources of TFA
302 isomers are supplied from different sources - not only from PHVOs, but also from deodorisation
303 of vegetable oils and deep-frying at high temperatures (Chowdhury et al., 2014).
304
305 *3.2.1. Bread category*
306 The 'bread' category contained from 0.10% to 1.71% TFAs (of total FAs), with a mean of 0.5%
307 ±0.5%, and a median of 0.2%. The mean contents of *trans*-C18:1 and *trans*-C18:2 isomers in
308 these analysed samples were 0.24% and 0.21%, respectively. There were only two samples
309 where no *trans*-C18:1 isomers were found. It is evident that in samples with more than 1%
310 TFAs, the amount of *trans*-C18:1 tended to predominate over *trans*-C18:2. In the samples with
311 <1% TFAs, there was no difference between these two C18 isomers. Among the *trans*-C18:2
312 isomers, *t*/*c*-C18:2 and *c*/*t*-C18:2 were in about equal amounts, while *t*/*t*-C18:2 was found
313 mainly in the samples with higher TFA content.
314
315 3.2.2. *Cookies/biscuits/wafers category*
316 High variability in TFA content within this category of cookies/biscuits/wafers was noted (up
317 to 31.03% of total FAs), which appeared to be due to different shortenings and fats used during

their manufacturing processes. The mean TFA content was 5.3%, with median of 1.4%. The level of 2% TFAs was exceeded in 35 of these 76 samples. In the samples with high total TFAs (>2%), the *trans*-C18:1 content (as 4.9% of total FAs) was higher than the *trans*-C18:2 content (Table 1).

In samples with >2% TFAs, the *trans*-C18:1 isomers from (*t*6-*t*8)-C18:1 to *t*13-C18:1 and *t*16-C18:1 were determined using an SP2560 capillary column (Figure 2). The *trans*-C18:1 profiles were similar for all of the products within each category (Table 2). Here, *t*9-C18:1 and *t*10-C18:1 predominated (42% of total *trans*-C18:1), followed by *t*11-C18:1 (16%) and the (*t*6-*t*8)-C18:1 group. According to previous findings, *t*9-C18:1 and *t*10-C18:1 are the usually major *trans*-C18:1 isomers in PHVOs, which correlated with increased risk of developing CVD (Aldai, de Renobales, Barron & Kramer, 2013).

3.2.3. Dough snacks category

The category of dough snacks consisted of *burek* (cheese pie; traditional food in some Balkan countries, which is also available in Slovenia). The TFA content ranged from 0.34% to 10.1% (of total FAs), with a mean of 1.8%, and a median of 1.5%. Five of these 21 'dough snacks' showed TFA contents >2%, with one notably surpassing this value (10.0%). This was a non-prepacked *burek* that was purchased at a stand, and therefore it did not have any list of ingredients.

Trans-C18:1 (1.4%) was more prevalent than *trans*-C18:2 (0.4%) (Table 1). In the samples with >2% TFAs, the C18:1 isomers were quantified (Figure 2, Table 2). The major *trans*-C18:1 isomer was *t*11-C18:1, at 29% of total *trans*-C18:1. The sum of the *t*9-C18:1 and *t*10-C18:1 isomers was approximately 36% of total *trans*-C18:1 FAs. As *t*11-C18:1 was the predominant *trans*-C18:1 isomer, these samples probably contained a proportion of ruminant fat, such as milk fat, cream and/or butter.

343

344 *3.2.4. Dough category*

345 The mean TFA content for the 'dough' category was 0.33% (of total FAs), with low variation
346 (range, 0.29% to 0.36%), and a median of 0.3%. All of these samples contained <2% TFAs.
347 The *trans*-C18:2 (0.26% of total FAs) were higher than the *trans*-C18:1 (0.07%). Among the
348 *trans*-C18:2 isomers, *c*/*t*-C18:2 and *t*/*c*-C18:2 were distributed relatively evenly, while *t*/*t*-
349 C18:2 was not detected. Abramovič et al. (2018) reported *t*/*t*-C18:2 mainly in margarine and
350 shortening samples with higher TFA content.

351

352 *3.2.5. t9/t11 ratio*

353 The *t*9/*t*11 ratio is important to assign the source of TFAs in processed foods. A *t*9/*t*11 ratio <1
354 is more related to ruminant fats, while a *t*9/*t*11 ratio >1 is more related to partly hydrogenated
355 fats (Abramovič et al., 2018). The *t*9/*t*11 ratio of 1.9 of the 'cookies/biscuits/wafers' category
356 indicated the inclusion of PHVOs, while in the 'dough snacks' category, the *t*9/*t*11 ratio of 0.72
357 was notably lower.

358 Vaccenic acid is the main TFA isomer in ruminant fats, and this has raised the question
359 whether its consumption has the same health risk implications as industrially produced TFAs.
360 Current data do not convincingly indicate adverse impacts of ruminant only TFAs or this *t*11-
361 C18:1 isomer on human health (De Souza et al., 2015). However, some data have also shown
362 potential benefits of ruminant TFAs (Ganguly & Pierce, 2015; Mills, Ross, Hill, Fitzgerald, &
363 Stanton, 2011). On the other hand, Stender (2015) argued that negative effects of ruminant
364 TFAs on LDL cholesterol are comparable with those of industrially produced TFAs. However,
365 *t*11-C18:1 is the only known dietary precursor of the *c*9, *t*11 conjugated linoleic acid (Field,
366 Blewett, Proctor & Vine, 2009). In three of the five samples of 'dough snacks' that contained
367 >2% TFAs, there were small amounts of conjugated linoleic acid (mean, 0.4% of total FAs).

368 The absence of higher risks of coronary heart disease associated with ruminant TFAs intake
369 compared to PHVOs intake might be due to lower levels of ruminant TFA intake (usually
370 <0.5% of total energy intake), to structural differences (not all isomers are the same), and/or to
371 some other factors (Ganguly & Pierce, 2015). Therefore, studies should not only focus on the
372 measurement of total TFAs in food products, but also on the identification of the specific TFA
373 isomers.
374
375 ***3.3 Atherogenocity and thrombogenocity indices***
376 Fatty acids can have positive or negative effects on atherosclerosis and coronary thrombosis,
377 which depend on the degree of saturation and the geometric positions of the double bonds. To
378 evaluate these effects, the AI and TI were calculated for these four categories of bakery
379 products. The AI and TI take into account the different effects that individual FAs can have on
380 human health, and in particular on the increasing incidence of atheroma or thrombus formation
381 (Ulbricht & Southgate, 1991). Fish is an example of a food that has low AI and TI, which is
382 provided by its high UFA content, and especially of *n*-3 FAs (Garaffo et al., 2011). AI <0.5 is
383 considered optimal. In the present study, relatively low AI and IT were determined for 'bread'
384 (0.14 to 2.01, 0.22 to 2.22, respectively), 'dough snacks' (0.28 to 1.93, 0.51 to 2.27,
385 respectively) and 'dough' (0.89 to 1.18, 1.58 to 2.36, respectively). Higher values of both AI
386 and TI were seen for the 'cookies/biscuits/wafers' category (0.09 to 4.50, 0.18 to 4.43,
387 respectively) (Figure 1). This is generally as expected, as the most pro-atherogenic and pro-
388 thrombogenetic FAs are at higher levels in the 'cookies/biscuits/wafers'.
389 In addition to SFAs, TFAs can have harmful effects on human health. This is why TFAs
390 levels were also used in the quantification of AI and TI. We thus also examined in particular
391 the samples with TFAs >2% of total FAs. Here, TFAs made notable contributions to AI and TI.
392 Indeed, when TFAs were removed from the calculation of AI and TI for

393 ‘cookies/biscuits/wafers’, these indices were reduced by 17% and 15%, respectively, and for
394 ‘dough snacks’, by 5% and 6%, respectively.
395
396 ***3.4 Multivariate analysis***
397 The relationships between the variables of the FA profiles were examined by factor analysis.
398 Before the factor analysis, and to show that the factor analysis was justified and the sample was
399 suitable for such processing, a Bartlett test was conducted (chi-squared = 3799.009, DF = 91,
400 P <0.001) and the Kaiser-Meyer-Olkin sampling coefficient was calculated (0.683). Here, the
401 communalities of 20 FA parameters were tested. These indicated the amount of variance that a
402 given variable shared with the extracted factors, and they should not be <0.50. Through this,
403 six parameters were eliminated from further processing: *trans*-C18:1 (% total TFAs); *trans*-
404 C18:1 (% total FAs); difference between TI with and without TFAs; difference between AI
405 with and without TFAs; AI without TFAs; *n*-3 FAs).
406 For factor analysis using the rest of the parameters, we selected those that carried the
407 largest share of all of the information. The first five factors explained 93% of the total
408 variability: factor 1 (117 determinations, 14 parameters) accounted for 46%; factor 2 for 27%;
409 factor 3 for 8%; factor 4 for 7%; and factor 5 for 5%. All of the other factors together accounted
410 for 7% of the total variability. Table S1 of supplementary data gives the factor weights
411 expressed as correlation coefficients, where a higher weight indicates a parameter that was more
412 important for the specific factor.
413 Factor 1 mainly reflects parameters related to the different FA groups (SFAs, UFAs, *n*-
414 6, PUFAs), the indices (TI, AI, TI without TFAs) and the ratios (UFA/[SFA + TFA],
415 UFA/SFA). Considering the coefficients of correlation (*r*) (Table S1 of supplementary data),
416 TI without TFAs, SFAs, TI, UFAs and AI give the greatest weights to this factor. TFAs give

417 the greatest weight to factor 2, and the *trans*-C18:2 contents of total TFAs give the main weight
418 to factor 3. MUFA and *n*-6/*n*-3 ratio determine factors 4 and 5, respectively.

419 Linear discriminant analysis was performed to classify these categories of bakery
420 products on the basis of their FA parameters. Altogether, 14 parameters were included in the
421 analysis. Using LDA, the following parameters were selected as the most discriminating
422 variables: indices, such as AI, TI, TI without TFAs, TFA content (TFAs, *trans*-C18:2 of total
423 FAs) and parameters related to the ratios of UFAs and SFAs, while the rest of parameters were
424 less important. When the LDA was applied to the data (117 samples, 14 variables), three
425 discriminant functions were obtained. Function 1 explained 58%, function 2 explained 38% and
426 function 3 explained 4% of the total variance. The scores of the samples and parameters for the
427 first two functions are shown in the discriminant score plot in Figure 3. The LDA revealed four
428 clusters, with the 'dough' category positioned within the 'cookies/biscuits/wafers' category. As
429 can be seen, the categories 'bread' and 'dough snacks' were well separated from each other.
430 Overall, the accuracy of the placement of each sample into its corresponding category was
431 90.6%.

432 A group of variables included in function 1 was clearly revealed, positioned far from
433 the origin. This group included TI, MUFAs and *n*-6 FAs. These variables were positively and
434 negatively correlated with TI without TFAs and UFAs/SFAs, respectively. Function 2
435 essentially grouped the variables *trans*-C18:2 of total FAs and AI; on the opposite side of
436 function 2, there were UFAs/(SFAs + TFAs) and TFAs.

437 'Cookies/biscuits/wafers' was the most heterogeneous category, and is positioned near
438 to origin on the left side of Figure 3. Samples from the 'dough' category also lie on the left
439 lower side of Figure 3, where TI without TFAs and UFAs/SFAs are grouped. In contrast,
440 samples from the 'dough snacks' category lie on the right upper side of Figure 3, where the

variables AI, TI and *trans*-C18:2/FAs are. Samples from the 'bread' category are on the right lower side of Figure 3, where the variable UFAs/ (SFAs + TFAs) is.

3.5. Daily consumed trans fatty acids and health aspects

To provide further insights about importance of investigated sources of TFAs intakes, we also calculated daily consumed TFAs for different population groups (infants, adolescents, adults, elderly) in Slovenia. EFSA's food consumption data for Slovenia was used to define typical daily food consumption for specific food category. As shown in Table 3, 'cookies/biscuits/wafers' were most important source of TFAs among all the investigated food categories, with mean daily consumed TFAs up to 0.49 g per day in adolescents. Considerably lower daily consumed TFAs in this category was calculated for other population groups. This trend is in line with results of nationally representative study of the dietary intake of TFAs in the Slovenian population (Zupanič et al., 2021), where for biscuits higher TFAs intakes were identified for adolescents, in comparison with adults and elderly. However, it should be noted that existing initiatives on the use of TFAs in foods have apparently quite successfully lowered the intake of industrial TFAs, as total dietary intake of industrial TFAs is relatively low (Zupanič et al., 2021). It should be also mentioned that even if the mean daily consumed TFAs are low, specific population groups with a preference for certain foods (e.g., low income groups) could consume TFAs in amounts that significantly increase their risk of CVD. The WHO recommends consumption of no more than 2.2 g TFAs per day for an adult consuming 2000 kcal per day (WHO, 2018). In Slovenia the mean daily consumed TFAs are below 2.2 g, while the maximal daily consumed TFAs in the adolescent group is above 2.2 g, but only for 'cookies/biscuits/wafers' category. These data indicates that we can exceed the WHO recommendation with the consumption of some foods with high TFAs content. Since not all sources of TFAs were included, the intake of TFAs could be underestimated, especially in

466 certain age groups, such as adolescents. To avoid this situation in the future, Slovenian
467 government further introduced regulatory limit of the amount of TFAs in foods (to 2 g of
468 industrial TFAs per 100 g of total fats in the food product) (-Uradni list, 2018) and this is
469 expected to further reduce dietary intake of TFAs.

470 Several studies have been performed to determine any connections between TFAs and
471 different health outcomes. Among these, Koba et al. (2019) showed that in Japanese men, total
472 C18:2 TFAs were significantly higher in an acute coronary syndrome group, than in the control
473 group (Koba et al., 2019). In the present study, the highest total C18:2 TFAs reached 1.3%,
474 while the majority of the samples contained between 0% and 1% total C18:2 TFAs (Table 1).
475 Therefore, the total C18:2 TFAs reported here are not a concern for consumers. Among the
476 18:1 isomers, Koba et al. (2019) only reported significantly higher levels for *t*12-C18:1 in the
477 acute coronary syndrome group, compared to control group. In the present study, *t*12-C18:1
478 represented a minor share, with a mean of 1% in 'cookies/biscuits/wafers' (Fig. 2A), and <0.4%
479 in 'dough snacks' (Fig. 2B). The literature data suggest that *t*9-C18:1 might be associated with
480 depression and that *t*10-C18:1 is associated with an increase in triglycerides (Liu et al. 2019).
481 Elaidic acid is highest in 'cookies/biscuits/wafers' here, as mainly the C18:1 isomer, followed
482 by *t*10-C18:1.

483 Data from the 1999-2000 NHANES study among the US population were used to
484 explain the connection between TFA isomers in plasma and different causes of mortality. The
485 results indicated that there is a relation between higher plasma *t*9-C18:1 levels and higher risk
486 of all-cause mortality (Li et al., 2017). For the connection between *t*9-C18:1 plasma levels and
487 CVD, data from two cycles of the NHANES study (1999-2000; 2009-2010) showed that high
488 plasma *t*9-C18:1 levels indicate higher CVD risk (Zhang, Yang, Hu, Li, Zhong & Huang, 2018),
489 as well as higher risk of mortality related to CVD, as shown in the 1999-2000 NHANES study
490 (Li et al., 2017).

491 We should also consider some of the limitations here. Standards could not be obtained
492 for all of the reported TFA isomers, so for their quantification, approaches described in the
493 literature were used. A further limitation of the study is that the AI and TI calculations were
494 carried out including previously reported coefficients for different types of FAs, although
495 additional clinical research would be required to determine whether those actually correspond
496 well to risk factors for development of CVD.
497
498 **4. Conclusion**
499
500 The results of the present study suggest that the FA composition of bakery products in the
501 Slovenian food supply should be improved by replacing atherogenic TFAs and SFAs with
502 beneficial FAs, to avoid adverse effects on human health. Among investigated food categories
503 'cookies/biscuits/wafers' were the most important source of TFAs. The mean daily consumed
504 TFAs are low, however the maximal daily consumed TFAs in the adolescent group is above
505 the WHO recommendation. These data are important for informing policy decisions further for
506 the reduction of TFA and SFA intakes, both at the national and global level. It should be noted
507 that even at very low TFA intake, plasma TFA levels remain significantly associated with serum
508 lipid and lipoprotein concentrations, which confirms that the intake of TFAs should be as low
509 as possible, within the context of a nutritionally adequate diet. These data also already support
510 the recent introduction of a legal limit for TFA content in foodstuffs in Slovenia, which has
511 been fully implemented since April 2019 (Uradni list, 2018).
512
513 **Declaration of Competing Interest**
514 The authors declare that they have no known competing financial interests or personal
515 relationships that could have appeared to influence the work reported in this paper.

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696

697 **Table 1.** Contents of total *trans*-fatty acids (TFAs), *trans*-C18:1 and *trans*-C18:2, and

698 *t*9/*t*11 ratio in the samples with >2% TFAs (n = 37). Data are means ±standard deviation.

Food category	Sample	*Trans*-fatty acid composition (% total FAs)			*t*9/*t*11 ratio	Labelled PHVO	Prepacked
		Total TFAs	*trans*-C18:1	*trans*-C18:2			
Cookies/biscuits/ wafers	TF-01	29.64 ±0.05	28.39 ±0.18	1.26 ±0.19	1.03	/	Yes
	TF-11	17.67 ±0.24	16.82 ±0.26	0.85 ±0.01	1.09	Yes	Yes
	TF-12	31.03 ±0.10	29.73 ±0.09	1.30 ±0.01	0.95	/	Yes
	TF-13	3.76 ±0.06	3.36 ±0.06	0.40 ±0.00	2.43	Yes	Yes
	TF-14	18.32 ±0.06	17.51 ±0.06	0.82 ±0.05	1.09	Yes	Yes
	TF-15	15.34 ±0.07	14.80 ±0.07	0.54 ±0.00	1.01	/	Yes
	TF-16	14.85 ±0.43	14.07 ±0.42	0.78 ±0.02	0.83	/	Yes
	TF-19	18.84 ±0.03	18.27 ±0.01	0.57 ±0.04	1.15	/	Yes
	TF-20	17.50 ±0.02	17.00 ±0.03	0.50 ±0.02	1.22	/	Yes
	TF-21	17.30 ±0.11	16.69 ±0.14	0.61 ±0.03	1.39	/	Yes
	TF-22	11.52 ±0.25	10.64 ±0.21	0.88 ±0.08	1.10	/	Yes
	TF-36	13.95 ±0.37	13.07 ±0.40	0.88 ±0.48	0.92	Yes	Yes
	TF-42	16.94 ±0.02	16.43 ±0.04	0.51 ±0.02	1.22	/	Yes
	TF-43	9.09 ±0.13	8.37 ±0.15	0.72 ±0.03	2.49	/	Yes
	TF-45	17.39 ±0.11	16.86 ±0.08	0.53 ±0.03	1.21	/	Yes
	TF-47	10.84 ±0.02	10.42 ±0.03	0.42 ±0.04	1.29	/	Yes
	TF-48	12.56 ±1.22	12.01 ±1.16	0.55 ±0.06	1.40	/	Yes
	TF-49	10.98 ±0.07	10.38 ±0.02	0.60 ±0.07	1.07	/	Yes
	TF-64	4.08 ±0.10	3.67 ±0.10	0.42 ±0.01	0.00	Yes	Yes
	TF-65	3.07 ±0.01	2.72 ±0.05	0.35 ±0.00	2.05	/	Yes
	TF-66	4.90 ±0.20	4.52 ±0.14	0.38 ±0.01	2.72	Yes	Yes
	TF-162	7.91 ±0.05	7.61 ±0.53	0.30 ±0.00	4.75	N/A	No
	TF-164	7.00 ±0.17	6.51 ±0.01	0.49 ±0.00	5.89	N/A	No
	TF-166	2.11 ±0.02	1.61 ±0.01	0.50 ±0.00	0.09	N/A	No
	TF-173	5.13 ±0.14	5.13 ±0.02	0.00 ±0.00	4.60	N/A	No
	TF-189	2.23 ±0.10	1.83 ±0.01	0.40 ±0.01	0.34	N/A	No
	TF-190	2.10 ±0.11	1.66 ±0.01	0.44 ±0.00	0.15	N/A	No
	TF-204	6.83 ±0.03	6.27 ±0.05	0.56 ±0.00	5.79	N/A	No
	TF-208	9.31 ±0.02	8.99 ±0.11	0.32 ±0.00	5.61	N/A	No
	TF-210	2.32 ±0.54	1.88 ±0.07	0.44 ±0.03	0.06	N/A	No
	TF-211	6.21 ±0.01	5.98 ±0.20	0.23 ±0.03	5.28	N/A	No
	TF-224	8.47 ±0.01	8.11 ±0.02	0.36 ±0.01	4.93	N/A	No
	TF-228	5.94 ±0.07	5.73 ±0.02	0.22 ±0.00	5.32	N/A	No
	TF-231	6.74 ±0.05	6.41 ±0.20	0.33 ±0.00	0.00	N/A	No
	TF-237	6.84 ±0.06	6.43 ±0.03	0.41 ±0.01	5.74	N/A	No

Dough snacks	TF-203	10.05 ±0.05	9.40 ±0.17	0.65 ±0.01	1.43	N/A	No
	TF-205	2.30 ±0.03	1.89 ±0.05	0.41 ±0.00	0.00	N/A	No
	TF-227	2.11 ±0.02	1.59 ±0.05	0.52 ±0.01	3.00	N/A	No
	TF-238	2.22 ±0.18	1.72 ±0.03	0.50 ±0.03	1.59	N/A	No
	TF-244	2.12 ±0.05	1.73 ±0.06	0.38 ±0.01	0.00	N/A	No

699 PHVO, partially hydrogenated vegetable oil

700 N/A, not applicable

701

Table 2. Contents of individual *trans*-C18:1 isomers (% of total *trans*-C18:1 isomers) and individual *trans*-C18:2 isomers in samples with >2% TFAs (n = 37).

Food category	Sample	C18:1 (% total *trans*-C18:1)[a]							C18:2 (% total *trans*-C18:2)[b]		
		(*t*6-*t*8)-	*t*9-	*t*10-	*t*11-	*t*12-	(*t*13-*t*14)-	*t*16-	c/t-	t/c-	t/t-
Cookies/biscuits/ wafers	TF-01	23.7	14.3	14.5	13.8	11.0	20.4	2.3	9.19	20.10	70.71
	TF-11	21.6	16.5	17.4	14.3	9.3	18.5	2.4	22.22	28.02	49.76
	TF-12	11.1	15.9	26.0	23.6	10.2	11.5	1.6	28.76	25.54	45.71
	TF-13	16.9	23.1	26.4	15.9	7.8	9.8	0.0	33.39	39.50	27.11
	TF-14	22.0	17.1	16.7	14.4	9.6	18.6	1.7	18.47	23.63	57.90
	TF-15	12.7	17.1	23.3	20.5	11.5	13.5	1.5	25.65	23.09	51.26
	TF-16	24.5	15.3	14.6	13.9	10.0	19.5	2.1	20.66	26.79	52.55
	TF-19	12.8	16.7	24.1	20.8	10.7	12.9	2.0	22.90	20.01	57.08
	TF-20	14.0	16.3	24.7	20.0	10.2	12.8	2.0	21.29	19.13	59.58
	TF-21	23.3	20.3	18.6	13.8	8.0	13.9	2.2	21.40	26.32	52.28
	TF-22	14.3	16.7	25.3	18.5	8.7	13.8	2.6	30.28	36.13	33.59
	TF-36	26.3	13.3	12.9	12.6	10.1	22.0	2.7	6.70	17.09	76.21
	TF-42	13.8	18.6	25.5	19.8	9.0	11.5	1.8	22.36	19.74	57.90
	TF-43	17.4	30.1	24.7	13.8	5.4	8.7	0.0	37.74	38.26	24.00
	TF-45	13.1	17.8	23.0	21.8	10.0	12.2	2.1	23.11	19.85	57.03
	TF-47	15.0	17.8	24.5	20.9	9.3	12.5	0.0	29.91	25.69	44.40
	TF-48	23.6	19.4	17.1	14.0	8.9	14.7	2.1	21.41	26.03	52.56
	TF-49	22.1	17.2	15.9	14.3	9.5	18.5	2.4	22.11	29.54	48.35
	TF-64	16.2	36.5	20.7	11.6	6.8	8.2	0.0	38.96	44.55	16.50
	TF-65	12.8	29.1	17.2	23.1	6.8	9.6	1.2	25.94	38.35	35.71
	TF-66	14.6	28.5	19.6	17.6	7.5	11.1	1.2	25.89	28.11	46.00
	TF-162	20.0	37.6	21.8	8.9	4.2	7.6	0.0	45.05	54.95	0.00
	TF-189	6.6	9.7	12.2	38.5	9.7	21.4	2.0	10.29	10.67	79.04
	TF-190	3.7	12.1	9.1	42.2	7.5	25.4	0.0	7.16	7.64	85.20
	TF-204	15.6	37.5	21.6	10.3	6.3	8.8	0.0	37.09	43.20	19.71
	TF-208	17.3	35.4	22.7	9.7	5.7	9.2	0.0	26.99	34.47	38.54
	TF-210	6.4	10.3	10.4	40.2	9.4	23.3	0.0	6.60	6.77	86.64
	TF-211	17.1	34.2	23.1	10.9	6.0	8.7	0.0	35.33	42.17	22.50
	TF-224	20.2	33.3	23.6	9.2	4.9	8.9	0.0	28.27	34.54	37.19
	TF-228	18.7	34.8	24.6	10.2	4.7	6.9	0.0	36.69	45.14	18.17
	TF-231	19.1	36.4	26.1	5.1	4.7	8.6	0.0	30.87	40.40	28.73
	TF-237	18.2	38.4	23.4	8.9	4.3	6.9	0.0	34.69	43.34	21.98
Dough snacks	TF-203	11.4	17.3	22.5	23.1	11.9	12.2	1.6	16.49	16.45	67.06
	TF-205	7.3	9.0	8.0	56.3	5.0	14.3	0.0	17.05	23.10	59.85

TF-227	17.6	27.4	18.4	18.8	6.3	11.6	0.0	35.86	48.60	15.54
TF-238	13.9	23.5	19.2	24.9	6.2	12.3	0.0	30.67	43.47	25.86
TF-244	6.4	11.2	12.4	42.7	6.9	14.5	6.0	12.25	12.97	74.78

704 [a] determined using a Supelco 2560 column

705 [b] determined using a BPX 70 column

706

707

708 **Table 3.** Daily amount of food consumption, TFAs content in food and daily consumed TFAs

709 for different population groups (infants, adolescents, adults, elderly) in Slovenia.

		Daily amount of food consumption	TFAs content in food		Daily consumed TFAs	
		g/day[a]	g/100 g of food[b]		g/day	
Category	**Population Group**		**Mean**	**Max**	**Mean**	**Max**
Bread	Infants	12.73	0.12	0.55	0.02	0.07
	Adolescents	108.29			0.13	0.60
	Adults	113.62			0.14	0.63
	Elderly	126.65			0.15	0.70
Cookies/biscuits/wafers	Infants	8.03	1.91	10.05	0.15	0.81
	Adolescents	25.52			0.49	2.57
	Adults	15.40			0.29	1.55
	Elderly	13.80			0.26	1.39
Dough snacks	Infants	0.30	0.27	1.83	0.00	0.01
	Adolescents	2.98			0.01	0.05
	Adults	1.75			0.00	0.03
	Elderly	0.87			0.00	0.02
Dough	Infants	0.29	0.12	0.15	0.00	0.00
	Adolescents	21.48			0.03	0.03
	Adults	14.76			0.02	0.02
	Elderly	4.81			0.01	0.01

710 TFA: *trans* fatty acid

711 [a] Source: EFSA Comprehensive European Food Consumption Database. (2021).

712 [b] The mean and maximal TFAs contents as we determined in different food categories.

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