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Cordelia Running
Purdue University, crunning@purdue.edu

John E. Hayes
The Pennsylvania State University, jeh40@psu.edu

Gregory R. Ziegler
The Pennsylvania State University, grz1@psu.edu

Degree of free fatty acid saturation influences chocolate rejection in human assessors

Cordelia A. Running[1,2,†], John E. Hayes[1,2], Gregory R. Ziegler[2*]

[1]Sensory Evaluation Center, [2]Department of Food Science, College of Agricultural Sciences, The Pennsylvania State University, University Park, PA 16802, United States

*Corresponding Author:

Gregory R. Ziegler

341 Erickson Food Science Building

University Park, PA 16802

United States

Tel: 814-863-2960

Fax: 814-863-6132

E-mail: grz1@psu.edu

† This work was performed while the author was a postdoctoral research associate at Penn State. She is currently a faculty member at Purdue University.

Keywords: oleogustus, chocolate, free fatty acids, non-esterified fatty acids, preference

Abstract

In foods, free fatty acids (FFA) traditionally have been viewed as contributing an odor, yet evidence has accumulated that FFA also contribute a unique taste ("oleogustus"). However, minimal work has been conducted using actual foods to test the contribution of FFA to taste preferences. We chose to investigate flavor, taste, and aroma contributions of added FFA in chocolate, as some commercial manufacturers already use lipolysis of triglycerides to generate unique profiles. We hypothesized small added concentrations of FFA would increase preferences for chocolate while higher added concentrations would decrease preferences. We also hypothesized a saturated fatty acid (stearic C18) would have a lesser effect than a monounsaturated (oleic C18:1), which would have a lesser effect than a polyunsaturated (linoleic C18:2) fatty acid. For each, paired preference tests were conducted for 10 concentrations (0.04% to 2.25%) of added FFA compared to control chocolate without added FFAs. Stearic acid was tested for flavor (tasting, nares open), while the unsaturated fatty acids were tested for both aroma (orthonasal only, no tasting) and taste (tasting with nares blocked to eliminate retronasal odor). We found no preference for any added FFA chocolate; however, rejection was observed independently for both taste and aroma of unsaturated fatty acids, with linoleic acid reaching rejection at lower concentrations than oleic acid. These data indicate degree of unsaturation influences rejection of both FFA aroma and taste in chocolate. Thus, alterations of FFA profiles in foods should be approached cautiously to avoid shifting concentrations of unsaturated fatty acids to hedonically unacceptable levels.

Keywords: oleogustus, chocolate, free fatty acids, non-esterified fatty acids, preference

Introduction

While lipids in foods are typically assumed to exist as triacylglycerols (where fatty acids are esterified to a glycerol backbone) many foods also contain meaningful levels of free fatty acids. These free fatty acids (FFA) have long been recognized as an important contributor to flavor in foods, including items such as dairy or meat products (Holland *et al.* 2005; Lindsay 2007; Neethling *et al.* 2016; Toldra and Flores 1998; Wong *et al.* 1975). Traditionally, FFA were believed to contribute to flavor strictly through aroma, either directly (mostly volatile, shorter chain FFA) or through the products of oxidative rancidity (mostly polyunsaturated FFA). However, recent evidence indicates humans and other mammals also have the ability to taste FFA, even when olfactory contributions to flavor are eliminated. The unique taste of FFA – "oleogustus" – has been proposed as a sixth prototypical taste (Running *et al.* 2015), and critically, the resultant percept appears to be unique and distinct from the textural contribution of triacylglycerols to food, and also from the well known odors associated with FFA.

To date, work on oleogustus in actual foods is lacking. Most prior studies on FFA taste have emphasized detection thresholds for pure FFA or used products with varying levels of triacylglycerols (Keller *et al.* 2011; Running *et al.* 2013; Stewart *et al.* 2010; Tucker *et al.* 2015). Further, almost all the prior work on oleogustus in humans has used oleic acid, and it is clear that sensitivity to and qualitative perception of oleogustus depends not only on overall concentration of the FFA but also on the structure of the FFA, including chain length and degree of unsaturation (Running *et al.* 2015; Running and Mattes 2014a; Running and Mattes 2014c). These differences in sensitivity and flavor depending on unsaturation are particularly important considering health messages emphasizing consumption of poly-unsaturated fats over saturated fats. As the flavor of fatty acids, whether through odor or taste, is often unpalatable, replacing saturated fats with unsaturated fats has implications for human willingness to comply with a particular diet.

Accordingly, we chose to investigate the contributions of FFA to preferences for chocolate, considering both taste and odor. Chocolate is a particularly relevant product in which to study oleogustus, as some highly commercially successful chocolate products intentionally use lipolysis as a means to obtain a unique flavor profile. For example, the Hershey's® company uses lipolyzed milkfat in many their chocolate products, which creates the characteristic sour or tangy Hershey® chocolate flavor that is very popular in the United States and simultaneously scorned by European chocolate consumers (Metz 2015; Moskin 2008). Likewise, the company ButterBuds® uses lipolysis to create numerous flavor additives from various fat products, including cream, cocoa butter, and olive oil (ButterBuds 2016). Thus, we decided to test how long chain fatty acids would contribute to the aroma and the taste of chocolate, within the context of oleogustus. Long chain fatty acids were selected because they are the dominant form of fat in chocolate, and also because these compounds are believed to contribute more strongly to the unique sensation of oleogustus, whereas shorter chain fatty acids are more sour in quality (Running *et al.* 2015). We selected 3 fatty acids – stearic, oleic, and linoleic – as these acids are matched for chain length (18 carbons) but differ in degree of unsaturation, with zero, one, and two double bonds respectively. Structures of these fatty acids are displayed in Figure 1. All three of these fatty acids are present in chocolate products, both in the esterified (as triacylglycerol) and non-esterified (as FFA) forms. Table 1 shows concentration ranges in 9 actual chocolates for these three fatty acids isolated in the non-esterified form (see (Perret *et al.* 2004) for original data).

We hypothesized that addition of free fatty acids would result in rejection of chocolate at higher concentrations, but that a preference might be observed at the lowest concentrations of added free fatty acids. Further, we hypothesized that degree of saturation would be related to rejection: linoleic would be rejected at lower concentrations than oleic acid, which would be rejected at lower concentrations than stearic acid. Finally, we hypothesized chocolate would be rejected at lower concentrations on the basis of aroma (orthonasal odor) versus taste (nose clipped) for oleic and linoleic acid; for this final hypothesis, stearic was not tested. This experiment also yields data on overall patterns of sensitivity and affective response to odor compared to taste of fatty acids in a real food system. Again, as most work to date has been conducted on thresholds rather than super-threshold perception, this work also expands knowledge of chemosensation of fatty acids differing by saturation.

Methods

Dark chocolate, made with deodorized cocoa butter to minimize endogenous levels of free fatty acids, was kindly donated by the Blommer Chocolate Company (East Greenville, PA). Three long chain fatty acids differing in degree of unsaturation were used: stearic (saturated C18), oleic (monounsaturated C18:1), and linoleic (polyunsaturated C18:2). These three fatty acids (from Sigma Aldrich) were dissolved in melted chocolate on a percent weight basis, as shown in Table 2, which also gives approximate molarities for these samples (actual molarity would depend on the density of the chocolate, which was not measured). This series of concentrations (0.04-2.5%, by one-fifth $\log_{10}$ steps) for FFA were selected based on prior work demonstrating human detection of taste of fatty acids in this range, as well as literature documenting FFA levels in chocolate that may occasionally range this high, at least collectively if not for an individual fatty acid (Perret *et al.* 2004; Running and Mattes 2014b; Running and Mattes 2014c). Chocolate was also served melted, approximately 43°C (110°F). This was done to minimize effects of adding liquid fatty acids (oleic and linoleic) on the texture of the final product. In recruitment and testing, the chocolate was described to participants as “warm chocolate sauce” so that expectations were congruent with a liquid product.

Participants were recruited from the Penn State campus and surrounding community. All participants provided informed, implied consent and were paid for their time. Study procedures were exempted from Institutional Review Board review by professional staff in the Penn State University Office of Research Protections under the wholesome foods/ approved food additives exemption in 45 CFR 46.101(b) (6). Per this exemption, we obtained “implied” consent through the initial screen of our CompuSense questionnaire, which gives an overview of the study ingredients and asks participants to click “Yes” they agree to participate or “No” they decline. No subjects declined. Eligibility requirements included: no food allergies, no known defects in smell or taste, between 18 and 55 years of age, no history of choking or difficulty swallowing, non-smoker, and no tongue/lip/cheek piercings. To reduce the total participant burden in terms of number of trials while still covering a wide concentration range of stimuli, each participant was randomized to one of two groups where they tasted either sample numbers 1,3,5,7, and 9 or 2,4,6,8 and 10 (see Table 2). These will be referred to as the “Odd” and “Even” groups, respectively. For the stearic acid test, there were 41 participants (12 men) in the Odd group and 37 participants (9 men) in the Even group. For oleic acid, there were 33 (13 men) in the Odd and 36 (8 men) in the Even groups.

For linoleic acid, there were 38 (8 men) in the Odd and 37 (18 men) in the Even groups. Additional details on the participants can be found in Supplemental Table 1.

Samples of ~4g were presented in 30mL cups labeled with random three digit blinding codes. Participants sat in individual sensory booths under a northern daylight illuminant (5000K LED) located directly overhead, and sample presentation order and data collection were conducted using Compusense® Cloud. Participants performed a "rejection threshold" task. This procedure gives participants an ascending series of test concentrations and controls, asking the participant to indicate their preference at each concentration. In our test, participants received samples in pairs, where each pair included a test sample (with added free fatty acid) and a control (no added free fatty acid). Across pairs, the concentration of the test sample increased in ascending order of added free fatty acid, so that any lingering aroma/taste of the free fatty acids would have minimal effect on the next sample pair. For each pair, participants indicated which sample they preferred in a forced choice task (2AFC preference) and why, in an open-ended text box. A 2-minute break, during which participants were instructed to rinse with room temperature reverse osmosis water, was enforced in between pairs.

For linoleic and oleic acids (but not steric acid), the preference test for each pair was administered twice: once for aroma only, in which participants were instructed to smell the samples and indicate their preference, and once for taste only, in which participants wore nose clips while tasting the pair and indicating preference. Prior work indicates the three fatty acids used in our experiment are not distinguishable from blanks when wearing nose clips (Bolton and Halpern 2010).All participants first smelled the samples, indicated preference, then received the samples again (with different three digit codes) to taste. Samples were presented with different three digit codes for the aroma and taste portions, so that participants would not be biased by their responses to the aroma preference question. Stearic acid was not tested in this manner, both because very minimal odor was noticed from the stearic acid in initial testing by our team, and because no difference in preference was observed for the stearic acid test conducted with nose open. Thus, we felt it would be a waste of resources and an undue burden on participants to give them added stearic acid in a nose closed condition, as it is very unlikely that a preference would emerge when orthonasal odor and taste were isolated as done for linoleic and oleic acids.

Baseline FFA concentration of the chocolate was measured by extracting the fat with petroleum ether, then titrating with 0.10 N KOH. More aggressive acidic digests of the sample were avoided in order to minimize generation of new FFA and mimic conditions more similar to the lipid that would accessible in the oral cavity. Measurements were made in triplicate.

Data were analyzed against the binomial distribution with alpha set at 0.05. Data were also analyzed for whether any group proportion crossed our existing definition of a rejection threshold, which requires 75% of participants to reject the test sample compared to the control – i.e., a chance adjusted threshold that is halfway between random responses (0.5) and perfect rejection (1.0) (see (Bakke *et al.* 2016; Harwood *et al.* 2012)). Finally, linear regression lines (using $\log_{10}$ values of concentration) were fit to the data. Linear regression was chosen over logistic regression due to the better fit for the regions displayed by our data. For clarity in the remainder of the manuscript, we operationally define "flavor" as in mouth sensation combining retronasal olfaction and taste (as in stearic acid test), "aroma" as the sensation from orthonasal stimulation only, and "taste" as the sensation arising from chocolate in the mouth when the nose is clipped (as in oleic and linoleic acid tests).

Results

The chocolate's baseline FFA concentration per titration was 0.79±0.06% as oleic acid equivalents. Results for the preference tests are summarized in Figure 2. For stearic acid, no preference or rejection was observed for flavor at any concentration (nose open, taste and retronasal olfaction combined), and the regression model did not show any evidence of a relationship between log concentration and group proportions (R^2=0.15, *p*=0.28).

For oleic acid, a pattern emerged with significant preference for the control over the added FFA sample, especially for aroma (orthonasal only). For aroma, the significant rejection (by binomial test, not rejection threshold) of added FFA at 0.040% (the lowest concentration, far left) was likely a false positive (type I error). Deleting this point results in better fit of the line (R^2=0.50, *p*=0.02 when all points are included, versus R^2=0.90, *p*=0.0001 when the 0.04% point is excluded). The regression model for aroma indicates a rejection threshold (75% rejecting) near ~1.29% added oleic acid, although we also note that if the pairs are considered independently, the 0.63% oleic acid sample reached criterion for a traditional binomial test for paired preference data (22 of 33 preferred the control; p = 0.04). For oleic acid taste (nose clipped), the regression model suggests significant rejection (binomial test) may begin just at or above 2.3% added oleic acid, however, this value should be interpreted very cautiously, as it is near the top of the range tested, and the model fit was relatively poor (R^2=0.29, *p*=0.11). A rejection threshold (75% rejection) for oleic acid would be well outside the range of realistic concentrations of oleic acid in chocolate.

For linoleic acid, the pattern of rejection with increasing added fatty acid is even more pronounced than for oleic acid. The regression model for the aroma data indicates a rejection threshold near~0.36% added linoleic acid (R^2=0.96, p<0.00001), and when considered individually, the 0.16% added linoleic acid sample reached criterion for a traditional binomial test for paired preference data (24 of 37 preferred the control; p = 0.049). For taste, these values are shifted to higher concentrations, but the regression model indicates a rejection threshold near 2.2% added linoleic acid, although the fit, while significant, is not as strong (R^2=0.65, p=0.005). Details for the fitted lines are given in Table 3.

Discussion

When chain length was constant, the degree of saturation of the added free fatty acid clearly influenced rejection for chocolate samples, with more unsaturation shifting rejection to lower concentrations. This was true both for smell (aroma) as expected, and for taste when the chocolate was eaten but olfactory input prevented via nose clips. Rejection was observed at lower concentrations for aroma (orthonasal smell only) than for taste for both oleic and linoleic acids. Conversely, the saturated fatty acid, stearic acid, did not show evidence of any influence on preference. Contrary to our hypothesis, low levels of fatty acids did not enhance preference for chocolate in blind testing, in spite of the existence of commercially available cocoa butter lipolysis products that are sold as flavor enhancers. The lack of a preference in our data could potentially be due to the fact that a baseline concentration of FFA was unavoidable in the control (0.79% in our product).

Patterns for perception of FFA observed in this study mirror previous results, with the mono-unsaturated FFA (oleic) less potent than poly-unsaturated FFA (linoleic) (Running and Mattes 2014a; Running and Mattes 2014c). The saturated FFA (stearic) had no contribution to flavor preference in the current study, and data are scarce for stearic acid in other work due to the solid nature of this lipid. Notably, the values calculated for rejection, either looking at significance or the rejection threshold definition, are generally above the measured concentrations of each of the individually tested FFA in previous reports (as seen in Table 1), except for the aroma of linoleic acid. European guidelines limit the concentration of FFA in cocoa butter to 1.75% (EEC 1973), but no restrictions are placed on the FFA concentration in the final chocolate product. Nonetheless, adding the baseline concentration of FFA in our chocolate to the values seen for rejection, total values for FFA for rejected concentrations of oleic and linoleic acid would fall around the 1.75% cutoff. The lack of rejection for stearic acid, even for concentrations well above the 1.75% cutoff, clearly indicates that fatty acid composition should be considered when using FFA concentration as a proxy for chocolate quality.

While some of the greater rejection of linoleic acid samples could be due to the accumulation of oxidation products over the course of the experiment, the high concentration of antioxidants in chocolates makes this a less likely contributor. Further, examination of the pattern of rejection over the course of the day reveals very linear cumulative rejection over time; if oxidative products were greatly contributing to rejection of linoleic acid samples, we would expect a non-linear relationship with relatively greater rejection in the latter part of the day compared to the earlier portion of the day (chart included in supplemental data).

Comparing aroma only to taste only regression lines, a better fit is consistently seen for aroma rejection. This is not particularly surprising, however, considering that work on oleogustus indicates very high variance in human perceived intensity of and sensitivity to free fatty acid tastes (Running *et al.* 2013). Many participants may not have perceived the fatty acid at all, leading to no preference, while others may have perceived the fatty acids at even the lowest concentration. As the goal of the current study was to test for preference in an actual food, data are unavailable for which participants could, or could not, detect the FFA. A higher proportion of discriminators would be expected at the higher concentrations of unsaturated FFA, as these concentrations led to rejection and rejection is implausible without detection. However, at the lower concentrations, at which no rejection was observed, participants could have either not detected the FFA or simple not cared about it.

Beyond the applicability of this work specifically to chocolate, the patterns of rejection are intriguing for work in the chemosensory field. Consistently, research demonstrates that rodents prefer long-chain fatty acids to controls, even in brief access tests (Fukuwatari *et al.* 2003; Gilbertson and Khan 2014; Tsuruta *et al.* 1999). Why rodents appear to prefer this taste sensation, yet humans reject it (at least at concentrations tested to date), is unclear. Our current study yet again demonstrates the human affect toward oleogustus, even in an actual food.

Conclusion

This study demonstrated that degree of unsaturation influences rejection of a chocolate with added FFA, with the polyunsaturated (linoleic) fatty acid being rejected by both taste and aroma at lower concentrations than the monounsaturated (oleic) fatty acid, and no rejection observed for the flavor of the saturated fatty acid. While the concentrations that lead to rejection were generally higher than may be expected in well-prepared, properly stored chocolate, the patterns of rejection by fatty acid structure should be considered when developing new products or when selectively breeding plants for particular fatty acid profiles. Further, as many health recommendations stress replacing dietary saturated fat with polyunsaturated fats, the greater rejection of polyunsaturated fatty acids, both by aroma and taste, could complicate implementation of diets with "healthier" fatty acid profiles.

Conflict of interest disclosure

CAR has no conflicts to declare. JEH and GRZ have received speaking or consulting fees from corporate clients in the food industry. Additionally, the Sensory Evaluation Center at Penn State routinely conducts taste tests for industrial clients to facilitate experiential learning for undergraduate and graduate students. None of these organizations have had any role in study conception, design or interpretation, or the decision to publish these data.

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Table 1: Concentrations of FFA in 9 commercial chocolate samples from Italy (% w/w)

	Minimum	Maximum	Mean
Total FFA	0.3%	2.3%	1.0%
Stearic	0.065% *(16.3%)*	0.80% *(58.0%)*	0.37% *(32.7%)*
Oleic	0.061% *(8.5%)*	0.34% *(43.0%)*	0.17% *(19.8%)*
Linoleic	0.023% *(3.1%)*	0.14% *(9.1%)*	0.059% *(6.3%)*

Values calculated from (Perret *et al.* 2004)
Values in parentheses are percent of total FFA

Table 2: Added fatty acids in chocolate

Number	Percent (w/w)	Approximate mM*		
		Stearic	Oleic	Linoleic
1	0.040%	1.8	1.8	1.8
2	0.063%	2.9	2.9	2.9
3	0.10%	4.5	4.6	4.6
4	0.16%	7.2	7.2	7.3
5	0.25%	11.2	11.3	11.4
6	0.40%	18.2	18.4	18.5
7	0.63%	28.7	28.9	29.1
8	1.0%	45.5	45.8	46.1
9	1.6%	71.7	72.2	72.7
10	2.5%	112.4	113.2	114.1

*Assumes density of chocolate is 1.3 kg/L

Table 3: Fitted lines from linear regression
(x: $\log_{10}$ Concentration, y: proportion preferring added FFA)

Fatty acid	Modality	R^2	*p-value*	Rejection* (significant)	Rejection† (threshold)
Linoleic	Taste	0.65	0.005	0.58%	2.19%
	Aroma	0.96	9.4e-7	0.14%	0.36%
Oleic	Taste	0.29	0.11	2.35%	NA
	Aroma	0.50	0.21	0.38%	1.76%
	Modified	0.90	1.0e-4	0.48%	1.29%
Stearic	Flavor	0.15	0.28	NA	NA

*Where the regression line crosses below the shaded area in Figure 2
†Where the regression line crosses the dotted rejection threshold line in Figure 2

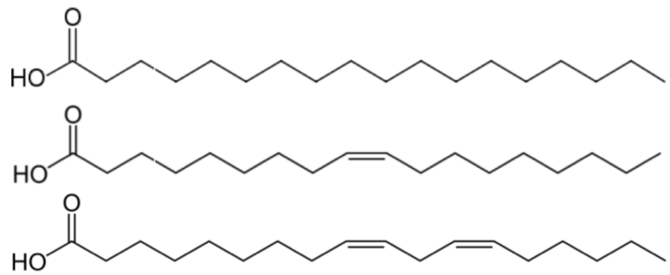


Figure 1: From top to bottom, structures of stearic, oleic, and linoleic acids.

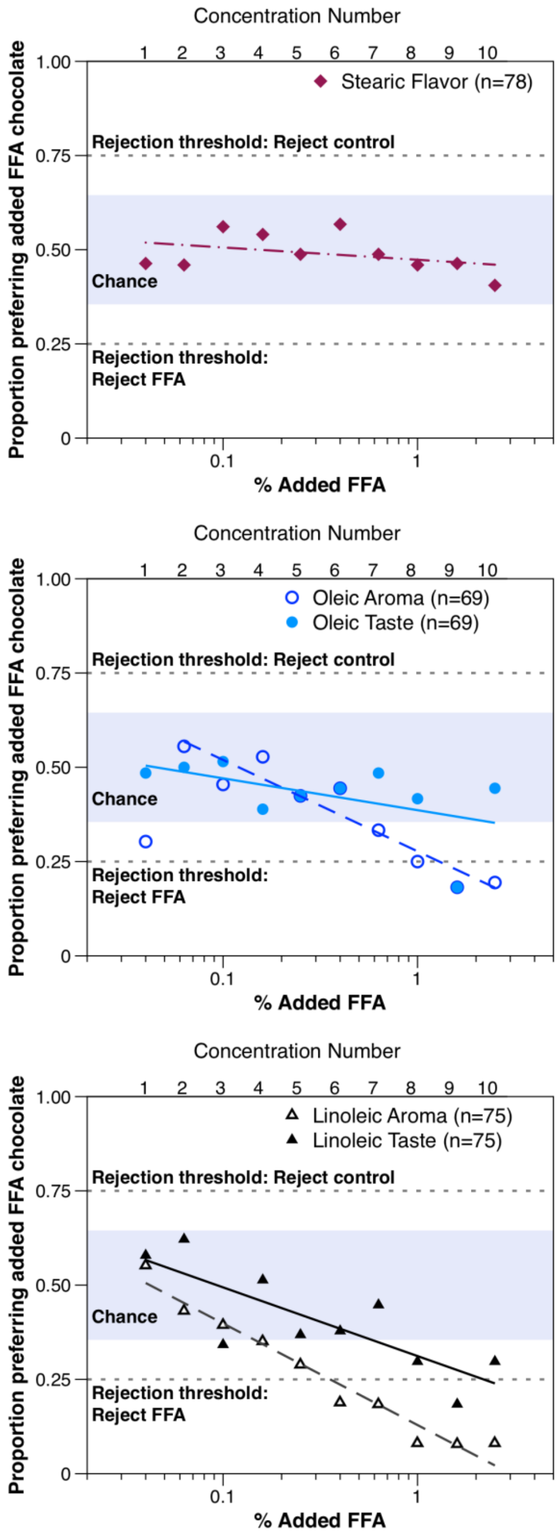


Figure 2: Proportions preferring added FFA to control chocolate. Actual chance proportion varies due to sample size differences at each level, but is labeled at 0.355-

0.645, which accurately categorizes all points in the dataset. Values for n are the total for the test, about half taste/smelled at each alternating concentration (1st,3rd,5th,7th,9th and 2nd,4th,6th,8th,10th)

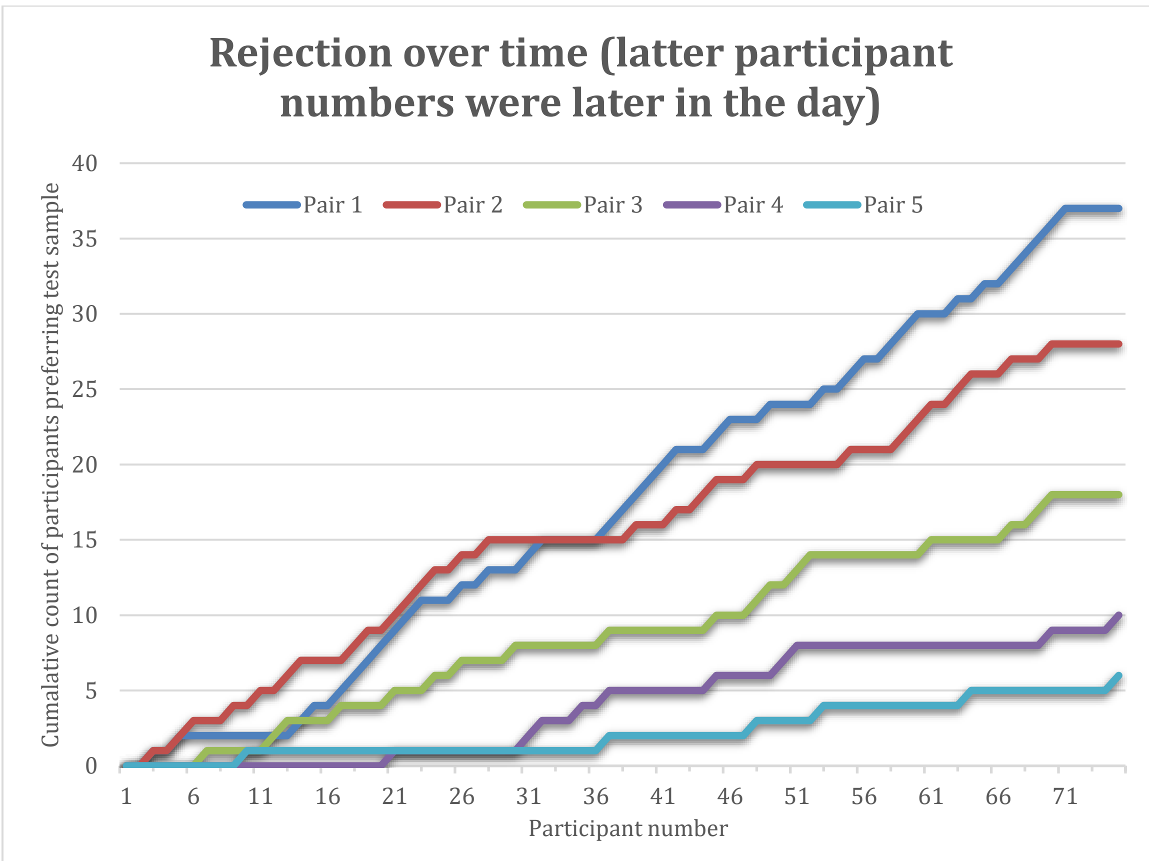
Supplemental Table 1: Participant characteristics

Age/Gender Summary	Count	Minimum Age	Maximum Age	Mean Age
Linoleic acid test total	75	18	61	31.1
Even Group total	37	18	61	31.1
Female	19	18	52	29.0
Male	18	19	61	33.4
Odd Group total	38	19	54	31.2
Female	30	19	54	33.4
Male	8	19	28	22.6
Oleic acid test total	69	18	54	34.3
Even Group total	36	19	54	33.4
Female	28	19	54	33.5
Male	8	19	47	33.0
Odd Group total	33	18	54	35.3
Female	20	18	54	37.4
Male	13	20	54	32.1
Stearic acid test total	80	13	55	32.1
Even Group total	38	18	55	33.7
Female	9	20	54	33.0
Male	29	18	55	33.9
Odd Group total	42	13	55	30.6
Female	12	20	52	27.9
Male	30	13	55	31.7

Race/Ethnic background

	Count
Linoleic acid test total	75
Even Group total	37
Hispanic	
Asian	1
Other	1
White	3
Non-Hispanic	
Asian	2
White	29
White and Native Hawaiian or Pacific Islander	1
Odd Group total	38
Hispanic	
White	2
Non-Hispanic	
Asian	3
White	33
Oleic acid test total	69
Even Group total	36
Non-Hispanic	
Asian	3
Other	1
White	32
Odd Group total	33
Non-Hispanic	
Native Alaskan or Native American	1
Asian	3
White	28
White and Asian	1
Stearic acid test total	78
Even Group total	37
Hispanic	

White	1
Non-Hispanic	
African	1
Asian	4
White	31
Odd Group total	41
Hispanic	
Native Hawaiian or Pacific Islander	1
Non-Hispanic	
Asian	6
White	34



Supplemental Figure 1: Rejection over time for linoleic acid pairs. Evidence that oxidation does not appear to be increasing rejection over time (lines should be more exponential in shape, rather than linear, if oxidation were increasing rejection over the course of the day).

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