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Title: Lipid oxidation during the beating of cake batter containing yellow pea (*Pisum sativum* L.) flour

Authors:

Svenja Krause, Eugenia Ayebea Asamoah, Gabrielle Moulin, Catherine Bonazzi, Barbara Rega*

Université Paris-Saclay, INRAE, AgroParisTech, UMR SayFood, 91300, Massy, France

* Corresponding author.

E-Mail addresses: svenja.krause@inrae.fr; eugenia.asamoah@inrae.fr;

gabrielle.moulin@agroparistech.fr; catherine.bonazzi@inrae.fr; barbara.rega@agroparistech.fr

Highlights:

- Extended beating of flour and oil favors the formation of volatile compounds
- 13- and 10-lipoxygenases are the main catalysts that oxidize fatty acids
- The degree of lipid oxidation depends on the batter microstructure and mixing time
- Reactivity increases in line with the surface of lipid droplets and air bubbles

Abstract:

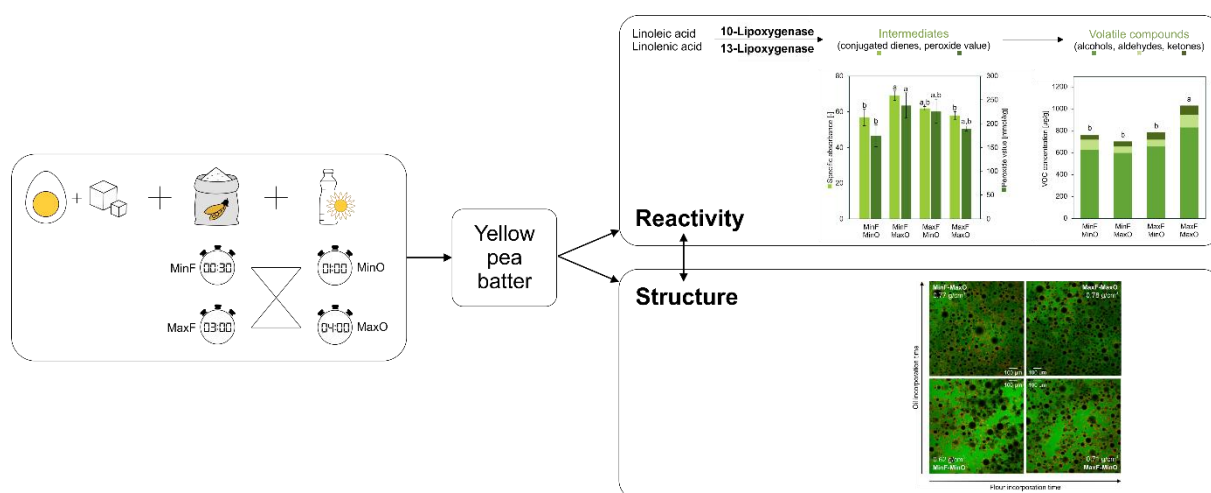
The aim of this study was to understand the impact of ingredients and processes on reactivity during sponge cake batter making using yellow pea flour. Numerous volatiles were identified, mainly originating from the degradation of linoleic and linolenic acid catalyzed by 10- and 13-lipoxygenases. The extent of lipid oxidation depended on the beating process, owing to the effect on local ingredient distribution and the exposure time of lipoxygenase to its substrates. Although prolonged beating after the addition of either flour or oil did not favor volatile formation, the latter led to elevated quantities of hydroperoxides. This intensified oxidation was assigned to an improved homogeneity and reduced size of lipid droplets and air cells. A sequential increase in both mixing times significantly increased the amount of volatiles, which could be attributed to a larger reaction interface and longer contact time between reactive ingredients. These results highlight the importance of parameter controls to limiting the generation of volatiles with odor activity.

Keywords: volatile organic compounds, confocal microscopy, structure, hydroperoxide, legume

Chemical compounds studied in this article:

1-Hexanol (PubChem CID: 8103); 1-Penten-3-ol (PubChem CID: 12020); Hexanal (PubChem CID: 6184); 3-Hexen-1-ol (PubChem CID: 10993); 2-Pentenal (PubChem CID: 12993); 1-Pentanol (PubChem CID: 6276); 1-Octen-3-ol (PubChem CID: 18827); 2-Octenal (PubChem CID: 16900); 3-Pentanone (PubChem CID: 7288); 2-Heptenal (PubChem CID: 5283316);

Graphical abstract:



1 Introduction

Growing interest in plant-based products to improve health, environmental sustainability and food security is continuously driving the incorporation of legumes in the human diet (Day, 2013; Hillen, & Garden Robinson, 2017). Among these, pea (*Pisum sativum* L.) represents a popular legume crop due to its abundant availability, low cost, attractive source of high-quality protein and low glycemic index (Dahl, Foster, & Tyler, 2012; Lam, Can Karaca, Tyler, & Nickerson, 2018; Marinangeli & Jones, 2011). Whole pea flour, in particular, can be considered an emerging ingredient for various food formulations because of its low degree of refining as opposed to isolated pea proteins.

Nonetheless, the applicability of such novel flour ingredients to replace traditional raw materials remains a challenge for the food industry because of their differing chemical composition which might alter reactivity during food processing, and hence product quality and safety. In the case of pea, exploitation of the benefits is especially limited because of the occurrence of inherent odor-active compounds and precursors to green-beany off-flavors (Krause, Keller, Hashemi, Descharles, Bonazzi, & Rega, 2021a). These volatile organic compounds typically originate from the lipoxygenase-catalyzed degradation of unsaturated fatty acids in the presence of oxygen (Hall *et al.*, 2017; Sessa & Rackis, 1977). Lipoxygenase (EC 1.13.11.12) is an iron-containing enzyme occurring in the raw material which introduces dioxygen into the carbon chain of fatty acids with a *cis,cis*-1,4-pentadiene system as found in linoleic and linolenic acid (Belitz, Grosch, & Schieberle, 2009). In the course of this reaction, hydroperoxides are formed and are subsequently cleaved into secondary structures, such as odorous alcohols, aldehydes and ketones, all with a probable impact on the sensory properties of the final food (Belitz *et al.*, 2009; Krause, Hashemi, Keller, Bonazzi, & Rega, 2021b).

Lipid oxidation plays a particular role in food systems that contain a complex mixture of different ingredients that undergo beating. This process is characteristic of the production of baked goods such as sponge cakes, which can be prepared using pea flour (Krause *et al.*, 2021a). The basis for these products is a foam obtained by whisking egg and sugar, into which flour particles are gently dispersed and oil droplets emulsified. During this mixing process, air and thus oxygen is entrapped in the batter matrix, which can trigger the oxidative degradation of unsaturated fatty acids (Krause *et al.*, 2021a; Maire, Rega, Cuvelier, Soto, & Giampaoli, 2013). The occurrence of this phenomenon when using pulse flours, and particularly pea flour, compared to traditional wheat flour was demonstrated in our previous publication (Krause *et al.*, 2021a). It is therefore of considerable importance to gain a

clearer understanding of the options available to control lipid oxidation during batter making so as to improve the sensory quality of cakes based on pulse flours. The aforementioned study also showed that the existing processing strategy of producing pea flour under nitrogen did not mitigate oxidative reactions but, on the contrary, it intensified them (Krause *et al.*, 2021a). A new approach was pursued subsequently, which consisted in varying the process conditions during batter preparation and thus identifying the importance of individual process steps in the development of lipid oxidation markers during batter making using yellow pea flour. To achieve this, the mixing times after the incorporation of flour and oil were separately or jointly extended and their impact on the release of intermediate and final lipid oxidation products was analyzed. At the same time, process-induced modifications to the batter microstructure were recorded so that their relevance to the degree of lipid oxidation could be determined.

2 Material and Methods

2.1 Ingredients

Yellow pea flour (23.8% protein, 47.6% starch, 8.8% moisture, 8.2% dietary fiber, 3.5% ash) was supplied by Cargill (Vilvoorde, Belgium). Sucrose was purchased from Tereos (Lille, France), sunflower oil from Lesieur (Asnières-sur-Seine, France) and whole pasteurized liquid eggs from Ovoteam (Locmine, France).

2.2 Chemicals

Furfural-d₄ (≥96%) and 2-methylpyrazine-d₆ (98.4%) were obtained from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada), hexanal-d₁₂ (≥96%), Celite® 545, iodine (≥99.8%) and aluminum oxide from Sigma Aldrich (Saint-Quentin-Fallavier, France), isopropanol, *n*-heptane, sodium thiosulfate pentahydrate, isooctane, glacial acetic acid, potassium iodide and iodine (0.02 M) from Carlo Erba (Val de Reuil, France) and DyLight 488 and Bodipy 655/676 from Thermo Fisher Scientific (Illkirch, France).

2.3 Purification of sunflower oil

In order to evidence the progress of lipid oxidation during batter preparation, the sunflower oil was purified by stripping its tocopherols as described by Pernin, Bosc, Soto, Le Roux, and Maillard (2019). In brief, 1 L sunflower oil was filtered three times at reduced pressure (960 mbar) through 200 g aluminum oxide activated overnight at 200 °C. The success of stripping was verified by recording the absorbance at 290-299 nm in isooctane using a fluorescence spectrophotometer (Cary Eclipse, Agilent Technologies, Les Ulis, France). The absence of absorbance bands within this wavelength range indicated the effective removal of tocopherols. The stripped oil was characterized by significantly lower peroxide values (2.31±0.26 mmol O₂/kg) and specific absorbances values for conjugated dienes at 234 nm (1.52±0.04) compared to the untreated oil (3.11±0.08 mmol O₂/kg and 2.62±0.14, respectively) because stripping purifies oils from polar compounds in general. Immediately after preparation, the stripped oil was stored in glass bottles under nitrogen and in the dark at -20 °C until use within two weeks.

2.4 Experimental design for batter preparation

Sponge cake batters were prepared according to the procedure published by Krause *et al.* (2021a). Egg (45% w/w) and sucrose (25% w/w) were thus whipped for 10 min into a stable foam, to

which non-sifted pea flour (25% w/w) was added gently within 90 s. After mixing for 30 s, sunflower oil was slowly incorporated within 15 s and the batter then beaten for 60 s (**Fig. 1**). This mixing protocol is denoted below as *MinF–MinO*.

Because of the known presence of relevant precursors or catalysts for lipid oxidation in flour and oil, it was deemed interesting to extend the mixing times after the incorporation of each ingredient. The aim was to alter not only the exposure time of the lipoxygenase contained in the flour to unsaturated fatty acids but also the amount of air, and thus oxygen, embedded in the batter, in order to identify the relevant process parameters that favor lipid oxidation and their effects on batter microstructure. A preliminary test was thus performed, during which beating after the addition of flour was prolonged from 30 s to 3 min (*MaxF*), followed by the extension of mixing after the addition of oil from 1 min to 4 min (*MaxO*). Over both periods, batter density was monitored in order to select maximal beating times after adding flour and oil that would be sufficiently long to modify air entrapment and exposure time of the enzyme to its substrates while concurrently avoiding collapse of the batter structure. Interestingly, batter density changed only slightly over both time periods when compared to *MinF–MinO*. For this reason, an experimental plan was set up in which four batters were prepared by crosslinking the maximal limits thus selected with the minimum conditions described in *MinF–MinO* (**Fig. 1**). Each batter was only made once because a preliminary test had confirmed insignificant variability ($p < 0.05$) between three independently prepared *MinF–MinO* batters in terms of the analyses described in the sections below. In order to prevent further oxidative reactions after making, the batters were frozen immediately at -20 °C under nitrogen in glass vessels sealed with septum-containing lids until analysis.

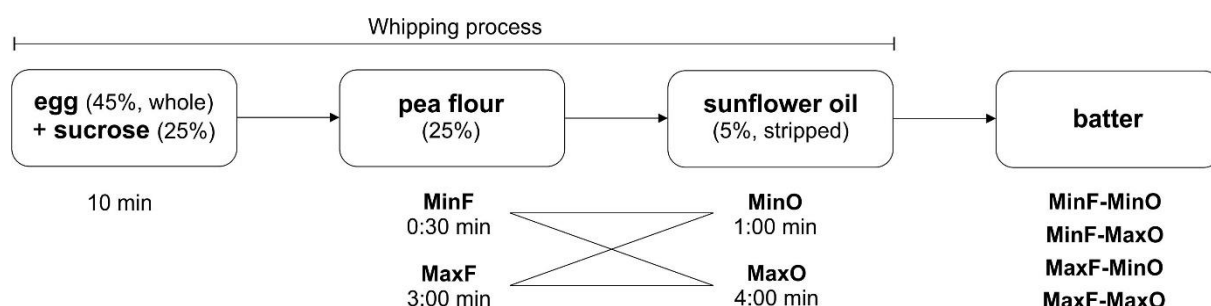


Fig. 1. Schematic illustration of batter preparation using different incorporation times for flour and oil.

2.5 Physical and chemical characterization of batters

2.5.1 Batter density

Batter density was determined in triplicate directly after preparation at room temperature by slowly drawing it up into 10 mL open-mouth syringes to prevent the destruction of gas cells. After weighing, density was calculated from the ratio of the mass of the batter to the volume of the syringes which was previously been defined using water.

2.5.2 Microstructure analysis by Confocal Laser Scanning Microscopy (CLSM)

The microstructure of the batters was analyzed using a Leica TC2 SP8 confocal laser scanning microscope (Wetzlar, Germany). 1 g freshly prepared product was stained with 3 µL of a mixture of DyLight 488 and Bodipy 665/676 (1/1 v/v) in order to the label proteins and lipids in green and red, respectively. After excitation at 488 nm (green) and 638 nm (red) using two lasers, the emitted fluorescence was detected in two channels within the wavelength ranges of 497-601 nm and 643-765 nm. Images were acquired at 10× magnification using a scan speed of 400 Hz, 70.7 µm pinhole and xyz scan mode.

2.5.3 pH value

To determine the pH, 2 g batter were dispersed in triplicate in 20 mL distilled water and the pH value recorded using a Sension+ PH3 meter (Hach, Loveland, CO, USA).

2.6 Analysis of lipid oxidation reaction

2.6.1 Lipoxygenase (LOX) activity

LOX activity of the pea flour was determined in triplicate by adapting the method described by Delcros, Rakotozafy, Boussard, Davidou, Porte, Potus, and Nicolas (1998). Briefly, 20 mL phosphate buffer (pH 7.5) was homogenized with 2 g flour and centrifuged, and 20-200 µL of the supernatant was mixed with linoleic acid in phosphate buffer (pH 6.5). LOX activity was expressed as nmol oxygen consumed per second.

2.6.2 Fatty acid profile, conjugated dienes and peroxide value

The oxidation status of the batters was monitored by measuring conjugated dienes, peroxide values and the profile of fatty acids after the extraction of fatty matter according to the accelerated solvent extraction procedure described by Krause *et al.* (2021a). In brief, the batters were pounded in a mortar with Celite® 545 (10/9 w/w), transferred into 34 mL stainless-steel extraction cells and

extracted for 25 min at 40 °C with a binary mixture of heptane and isopropanol (5/1 v/v) using a Dionex ASE 350 (Thermo Fisher Scientific, Waltham, MA, USA). After solvent evaporation at reduced pressure and 40 °C, the oil was deep-frozen at -20 °C under nitrogen until analysis of the reaction markers.

Conjugated dienes were determined in triplicate by adapting the method described by Roman, Heyd, Broyart, Castillo, and Maillard (2013). Firstly, dilutions of 10 mg extracted oil in isooctane (1:100-1:200) were prepared and the mixtures vortexed for 10 s. Absorbance was then recorded against isooctane in 1 cm quartz cuvettes at 234 nm using a Specord 210 (Analytik Jena, Savigny-le-Temple, France). The content in conjugated dienes was expressed as specific absorbance, which was defined as the absorbance of 1 g oil in 100 mL isooctane at 234 nm.

The **peroxide value** was analyzed in triplicate by iodometric titration according to Krause *et al.* (2021a). In brief, 200 mg extracted oil were dissolved in glacial acetic acid/isooctane (3/2 v/v, 16 mL), followed by the addition of saturated potassium iodide solution (1 mL) and distilled water (9 mL). Titration of the released iodine was performed using sodium thiosulfate (5 mM). The peroxide value was expressed as millimole of hydroperoxide per kg of oil.

The **profile of polyunsaturated fatty acids** was analyzed in triplicate as reported by Krause *et al.* (2021a). In short, 30 mg extracted oil were dissolved in petroleum ether (1 mL) containing heptadecanoic acid as standard, and the fatty acids were esterified at 52 °C for 40 s using NaOH in methanol (2 M, 200 µL). The reaction was stopped by HCl in methanol (1 M, 400 µL) and the fatty acid methyl esters (FAMES) present in 1 µL of the upper phase were separated on a TRACE GC Ultra gas chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a SP-2560 GC column (100 m × 250 µm × 0.2 µm, Supelco, Bellefonte, PA, USA). The oven program was set as follows: 100 °C isotherm (4 min); 100 °C to 200 °C at 5 °C/min; 200 °C isotherm (8 min); 200 °C to 250 °C at 5 °C/min, 250 °C isotherm (12 min). Helium carrier gas was used at a split flow of 448 mL/min. For detection, an ISQ single quadrupole mass spectrometer (Thermo Fisher Scientific) was operated at 250 °C, scanning masses from m/z 40 to 400. Compounds were identified by comparing the mass spectra and retention times with those of a standard mixture containing 37 compounds (Supelco) subjected to the same procedure. Relative FAME quantities were calculated using the internal standard.

2.6.3 Profiles of volatile organic compounds (VOCs)

VOCs were extracted by Headspace-Solid Phase Micro Extraction (HS-SPME) using the optimized procedure reported by Krause *et al.* (2021a). Two grams of batter were weighed in triplicate in 20 mL glass vials, spiked with 100 μ L of an aqueous solution containing 778 mg/L of the deuterated standard hexanal-d₁₂ and sealed using aluminum caps with PTFE/silicone septa. After overnight storage at 4 °C to equilibrate the standard within the sample, the vials were incubated at 50 °C for 18 min in a Triplus autosampler (Thermo Fisher Scientific). Volatiles were absorbed on a DVB/CAR/PDMS fiber (50/30 μ m, 2 cm, Supelco) for 42 min at 50 °C and finally desorbed for 2 min at 250 °C in a TRACE GC Ultra (10 mL/min) injector port.

The separation of VOCs was achieved on a ZB-wax capillary column (30 m \times 0.25 mm \times 0.5 μ m) by increasing the temperature from 40 °C (maintained for 5 min) to 240 °C at 4 °C/min. Helium was used as the carrier gas (1.2 mL min). Mass spectrometry was performed using an ISQ single quadrupole mass spectrometer (Thermo Fisher Scientific) operated in the EI mode at 70 eV, scanning in full scan (TIC, m/z 33-300) and SIM mode for selected molecules. VOCs were identified by matching (i) experimental mass spectra with MS libraries (NIST 08, Wiley 8) and (ii) by comparing experimental retention indices obtained using a series of *n*-alkanes with the reference values of the NIST database. Hexanal was quantified by comparing the peak areas with those of the internal standard hexanal-d₁₂. Similarly, relative quantities were determined for the remaining compounds (alcohols, aldehydes and ketones).

2.7 Statistical analysis

One-way analysis of variance (ANOVA) was performed and significant differences evaluated by Tukey's test at $p < 0.05$ using XLSTAT 2020.5.1 (Addinsoft, Paris, France).

3 Results and Discussion

3.1 VOCs as indicators of lipid oxidation during batter beating

Table 1 shows the volatile organic compounds extracted from the yellow pea batters prepared using different mixing times after flour and oil incorporation.

A total of 18 different VOCs were identified in the batter produced according to the standard protocol (*MinF–MinO*), the majority of which are alcohols (10), followed by ketones (5) and aldehydes (4). From a quantitative point of view, alcohols were observed as the dominant group of volatiles (626 µg/g), and thus accounted for 82% of the total VOC concentration (760 µg/g). The remaining fifth was mostly composed of aldehydes (91 µg/g), while ketones contributed only marginally (43 µg/g).

According to the general consensus in the literature, most of the alcohols, aldehydes and ketones detected were typical markers of the oxidative degradation of unsaturated fatty acids. Nevertheless, molecules such as ethanol also appeared at high concentrations (226 µg/g), which were assumed to have emerged from the flour or during fermentation reactions. Among the lipid oxidation-derived compounds, 1-penten-3-ol and 1-hexanol were the most abundant in *MinF–MinO*, each reaching values of around 160 µg/g (**Table 1**). The formation pathway for 1-penten-3-ol likely involves the oxidation of linolenic acid to 13-hydroperoxide, which in turn can be cleaved to form 1-penten-3-ol (Fisher, Grimes, & Fall, 2003; Gardner, Grove, & Salch, 1996; Salch, Grove, Takamura, & Gardner, 1995; Shen, Tieman, Jones, Taylor, Schmelz, Huffaker, Bies, Chen, & Klee, 2014). Alternatively, de Lumen, Stone, Kazeniac, and Forsythe (1978) proposed its generation from 16-hydroperoxide deriving from the same fatty acid, which could be isomerized enzymatically into 1-penten-3-one, followed by a reduction to 1-penten-3-ol catalyzed by an alcohol dehydrogenase (Ma, Boye, Azarnia, & Simpson, 2016). By contrast, 1-hexanol has been reported to arise from linoleic acid after peroxidation to the corresponding 13-hydroperoxide, from which hexanal can be released upon cleavage (Belitz *et al.*, 2009; Xu, Jin, Lan, Rao, & Chen, 2019). In a subsequent step, this aldehyde can be further transformed into 1-hexanol in the presence of an alcohol dehydrogenase (Belitz *et al.*, 2009; Jakobsen, Hansen, Christensen, Brockhoff, & Olsen, 1998). The occurrence of this consecutive reduction reaction in *MinF–MinO* was reflected by the lower relative concentration of hexanal compared to 1-hexanol (**Table 1**). Apart from the compounds mentioned above, other volatiles, similarly associated with the degradation of the 13-hydroperoxides described were also found. These included 3-hexen-1-ol (linolenic acid), 2-pentenal (linolenic acid) and 1-pentanol (linoleic acid), which

however accumulated in considerably smaller amounts (1-51 µg/g) (**Table 1**) (Frankel, 2012; Luning, Carey, Roozen, & Wichers, 1995; Przybylski & Eskin, 1995; Schaich, 2013). Furthermore, Sakouhi, Saadi, Herchi, Alibi, Luigi, and Boukhchina (2016) and Sánchez-Ortiz, Pérez, and Sanz (2013) had suggested that the ketone 3-pentanone, which totaled 32 µg/g, might have been produced through a similar reaction scheme.

Moreover, trace quantities of 1-octen-3-ol and 2-octenal were detected (1-6 µg/g), the synthesis of which is typically based on the oxidation of linoleic acid (Frankel, 2012; Kakumyan & Matsui, 2009). Unlike the previous volatiles, this oxidative breakdown takes place under the formation of an intermediate non-conjugated 10-hydroperoxide (Belitz *et al.*, 2009; Luning *et al.*, 1995).

Interestingly, oxidation products of monounsaturated oleic acid were also present in low concentrations (1-4 µg/g) (**Table 1**), comprising 2-heptanone, 1-octanol and 2-octanone (Cao, Zou, Deng, Fan, Li, Li, & Deng, 2014; Kochhar, 1996; Przybylski *et al.*, 1995).

In our previous publication on the ability of diverse pulse flours to generate VOCs during sponge cake making (Krause *et al.*, 2021a), we reported a significantly higher susceptibility of pulse flours to oxidative deterioration compared to wheat flour, which is traditionally used in bakery products. We postulated that LOX is the main catalyst of lipid oxidation during batter beating when using pulse flours, which could be attributed to a positive correlation between enzyme activity and VOC formation: while the sum of oxidation-derived alcohols, aldehydes and alcohols was negligible (<1 µg/g) in batters made with wheat flour, in which LOX was practically inactive (<20 nkat/g), those in batters based on flours from chickpeas, lentils or peas were several hundred higher because of LOX activities of up to 4350 nkat/g. In the present study, an average LOX activity of 5379 nkat/g yellow pea flour as well as a total relative VOC amount of 760 µg/g were determined, of which around 70% were assumed to most likely originate from lipid oxidation. This corresponded to a concentration of typical oxidation volatiles that was more than 500 times higher compared to the wheat flour batter of our previous study (Krause *et al.*, 2021a). It was thus hypothesized that LOX might likewise have played a crucial role in VOC development through fatty acid breakdown in the present yellow pea batter. This enzyme targets the *cis,cis*-1,4-pentadiene system of polyunsaturated fatty acids, such as linoleic and linolenic acid, thereby introducing dioxygen into the carbon chain to form the corresponding hydroperoxides. In accordance with Krause *et al.* (2021a), the oxygen solubilized in the batter during beating seemed to

275 be sufficient to trigger an instant reaction as soon as the dry ingredients were dispersed in the moist
276 batter matrix.

277 Pea may contain three, four or five isoenzymes of LOX, which were grouped by Fischer,
278 Cachon, and Cayot (2020) in one heat-stable LOX with a pH optimum above 8 and 13-regiospecificity
279 (LOX-1) as well as three heat-labile LOX with optimal activity at pH 6-7 but different regiospecificities
280 of 9, 10 and 13 (LOX-3, LOX-5 and LOX-2, respectively). The remaining LOX-4 has not yet been
281 identified. Owing to the quantitative dominance of 1-hexanol and 1-penten-3-ol in pea batter, which
282 has been shown to originate from the 13-hydroperoxides of linoleic and linolenic acids, it is reasoned
283 that the activity of a 13-LOX was of major importance. Because the pH values of the batters were
284 around 7.2 (**Table 3**), it could be imagined that LOX-1 and/or LOX-2 were active. This was further
285 corroborated by the presence of traces of other molecules at the same time, which also arose from
286 these 13-hydroperoxides. On the other hand, however, the occurrence of volatiles formed *via* 10-
287 hydroperoxides might also indicate the catalysis of fatty acid oxidation by a 10-LOX (LOX-5).

288 Although LOX activity is generally assumed to be the main driver for lipid oxidation during
289 batter beating, small amounts of VOCs arising from oleic acid were monitored. Based on the lack of a
290 *cis,cis*-1,4-pentadiene system in this fatty acid, which is a necessary requirement for LOX catalysis, it
291 was concluded that non-enzymatic lipid oxidation had taken place, but only to a limited extent.
292 Ongoing autoxidation was supported by the presence of 2-heptenal (**Table 1**), which has been
293 reported to arise from linoleic acid 12-hydroperoxide. To date, no 12-LOX has been detected in pea,
294 so the enzymatically-controlled formation of 12-hydroperoxide, and hence 2-heptenal could be ruled
295 out.

296 **Table 1.** Occurrence of volatile organic compounds [$\mu\text{g/g}$ batter] identified in yellow pea batters prepared using different mixing times after the addition of flour
 297 and oil (mean \pm SD, $n = 3$). Relative amounts are estimated based on the internal deuterated standard hexanal- d_{12} .

Compound	Selected ion (<i>m/z</i>)	Experimental retention index	Reference retention index ¹	Relative concentration of volatile organic compounds [µg/g batter]			
				MinF–MinO	MaxF–MinO	MinF–MaxO	MaxF–MaxO
Alcohols							
Ethanol	45	944	942	225.94 ± 18.32 ^a	214.26 ± 47.84 ^a	237.26 ± 55.72 ^a	248.86 ± 15.55 ^a
1-Propanol	59	1047	1049	5.59 ± 0.39 ^b	5.75 ± 0.83 ^b	5.11 ± 0.48 ^b	7.56 ± 0.10 ^a
1-Butanol	56	1153	1147	4.47 ± 0.49 ^a	2.76 ± 0.65 ^b	2.73 ± 0.48 ^b	4.26 ± 0.08 ^a
1-Pentanol	70	1257	1255	51.19 ± 3.66 ^b	44.31 ± 6.68 ^b	41.42 ± 4.93 ^b	64.06 ± 1.20 ^a
1-Hexanol	69	1360	1360	159.36 ± 8.20 ^{a,b}	131.09 ± 22.66 ^b	122.56 ± 15.65 ^b	190.28 ± 2.62 ^a
1-Octanol	56	1567	1569	0.83 ± 0.04 ^b	0.78 ± 0.13 ^b	0.70 ± 0.10 ^b	1.23 ± 0.03 ^a
1-Penten-3-ol	57	1169	1165	161.16 ± 15.30 ^c	234.98 ± 20.27 ^b	170.89 ± 18.14 ^c	287.72 ± 9.86 ^a
1-Octen-3-ol	99	1457	1458	5.65 ± 0.35 ^a	4.42 ± 0.45 ^b	4.34 ± 0.65 ^b	6.64 ± 0.19 ^a
3-Pentanol	59	1047	1049	10.64 ± 0.96 ^b	15.61 ± 2.83 ^{a,b}	11.23 ± 2.75 ^b	17.56 ± 0.83 ^a
3-Hexen-1-ol	82	1392	1394	1.47 ± 0.17 ^a	1.48 ± 0.34 ^a	1.64 ± 0.27 ^a	1.79 ± 0.11 ^a
Total				626.28 ± 47.89 ^b	655.44 ± 102.68 ^{a,b}	597.88 ± 99.16 ^b	829.97 ± 30.57 ^a
Aldehydes							
Hexanal	56	1091	1091	83.43 ± 3.79 ^b	55.36 ± 2.03 ^c	50.22 ± 3.89 ^c	104.25 ± 12.35 ^a
2-Pentenal	84	1137	1132	2.76 ± 0.31 ^c	4.24 ± 0.04 ^b	2.83 ± 0.51 ^c	5.83 ± 0.77 ^a
2-Heptenal	83	1332	1332	3.79 ± 0.24 ^b	2.69 ± 0.35 ^c	2.72 ± 0.20 ^c	5.47 ± 0.26 ^a
2-Octenal	70	1437	1437	1.15 ± 0.04 ^b	0.85 ± 0.08 ^c	0.79 ± 0.07 ^c	1.54 ± 0.16 ^a
Total				91.13 ± 4.38 ^b	63.13 ± 2.49 ^c	56.57 ± 4.67 ^c	117.08 ± 13.54 ^a
Ketones							
2-Butanone	72	911	900	1.77 ± 0.13 ^b	1.97 ± 0.13 ^b	1.65 ± 0.21 ^b	2.51 ± 0.14 ^a
2-Heptanone	58	1189	1185	3.68 ± 0.23 ^{b,c}	4.69 ± 0.43 ^b	3.52 ± 0.53 ^c	6.50 ± 0.28 ^a
2-Octanone	58	1292	1297	2.00 ± 0.09 ^b	2.40 ± 0.24 ^b	1.99 ± 0.36 ^b	3.30 ± 0.06 ^a
3-Pentanone	86	985	986	32.09 ± 2.51 ^c	54.48 ± 4.46 ^b	37.54 ± 3.77 ^c	64.28 ± 1.70 ^a
3-Octanone	99	1261	1261	3.22 ± 0.22 ^b	3.07 ± 0.44 ^b	2.68 ± 0.42 ^b	4.26 ± 0.04 ^a
Total				42.76 ± 3.18 ^c	66.60 ± 5.70 ^b	47.39 ± 5.29 ^c	80.85 ± 2.23 ^a
Total				760.17 ± 55.46 ^b	785.17 ± 110.89 ^b	701.84 ± 109.12 ^b	1027.90 ± 46.35 ^a

298 Different letters within a column indicate significantly different means ($p < 0.05$).

299 ¹ National Institute of Standards and Technology (NIST), 2021

3.2 Importance of mixing time on VOC quantities

As shown in **Table 1**, extending mixing times after the addition of either flour or oil from 30 s to 3 min (*MaxF–MinO*) and from 1 to 4 min (*MinF–MaxO*), respectively, as well as their combined extension (*MaxF–MaxO*) led to the release of identical volatile compounds but in different quantities.

In general, the individual extensions of either flour or oil beating times by 2.5 or 3 min, respectively, resulted in batters characterized by total VOC concentrations that did not differ significantly from that determined in *MinF–MinO* (785 µg/g and 702 µg/g vs. 760 µg/g). Apart from this, the percentage distribution of volatile levels between the chemical classes of alcohols, aldehydes and ketones remained almost constant. It was thus deduced that both mixing times were insufficient to drive the formation of volatile compounds. However, closer inspection of *MaxF–MinO* revealed that molecules known to originate from the oxidative degradation of linolenic acid (i.e. 1-penten-3-ol and 2-pentenal) appeared to be formed in increasing quantities (**Table 1**). Considering the proportions of the individual ingredients, as well as their fat content and fatty acid profile, it emerged that linolenic acid mainly derived from the egg and flour. This indicated that extending the mixing time after adding the flour increased the probability of contact between the enzyme and linolenic acid, thus leading to a higher proportion of linolenic acid-derived volatiles. This assumption was corroborated by non-significant differences in the levels of linolenic acid oxidation markers in *MinF–MaxO* compared to *MinF–MinO* (**Table 2**). During the short mixing after flour addition (30 s), competition for the active site of the LOX only occurred between the linoleic and linolenic acids arising from the egg and flour. The subsequent incorporation of oil rich in linoleic acid thus diluted the amount of linolenic acid in the batter. The extended exposure of LOX to this complex mixture of fatty acids therefore led to a similar VOC profile as that seen with the standard protocol.

A different picture emerged when both periods were extended at the same time, as the *MaxF–MaxO* batter thus produced was considerably enriched in all the lipid oxidation markers identified. In the case of alcohols, this increase was mainly ascribed to 1-penten-3-ol, reaching values around 288 µg/g, which corresponded to an approximately 80% increment compared to *MinF–MinO* (**Table 1**). Unlike the standard batter, this compound clearly dominated the profile of volatile compounds in *MaxF–MaxO*. Nevertheless, 1-hexanol and its precursor hexanal concurrently accumulated at high relative concentrations (190 and 104 µg/g, respectively), thus markedly contributing to the total VOC content. Interestingly, substantial increases in the amounts of minor compounds by up to 111% (2-

pentenal) were likewise recorded. These changes were not only assigned to molecules known to originate from the LOX-mediated oxidation of linoleic and linolenic acid, but also to those arising from the autoxidation of oleic acid. It was therefore concluded that the combined prolongation of mixing times after addition of both flour and oil, reaching a total of 5.5 min, was sufficient to prompt not only enzymatic but also non-enzymatic lipid oxidation.

3.3 Evolution of lipid oxidation as a function of mixing time

The GC-MS analysis of VOCs contained in the diverse batters revealed that extended mixing times for both flour and oil (*MaxF–MaxO*) favored molecule generation when compared to *MinF–MinO* (Section 3.2). By contrast, non-significant modifications were observed following the separate extension of beating times after the incorporation of either flour or oil (*MaxF–MinO* and *MinF–MaxO*, respectively). Volatile compounds, however, are the end products of fatty acid degradation, for which reason they are often described as secondary markers of oxidation. In fact, the oxidative breakdown of fatty acids takes place *via* intermediate products, or hydroperoxides, which are commonly referred to as primary lipid oxidation markers. Investigation of these intermediate structures can therefore serve to gain greater insight into the oxidation status of the batters and reveal differences that are not visible from the analysis of secondary oxidation products alone (Maire *et al.*, 2013).

Table 2. Peroxide value, specific absorbance of conjugated dienes and profile of polyunsaturated fatty acids of yellow pea batters prepared using different mixing times after the addition of flour and oil (mean \pm SD, $n = 3$).

Batter	Peroxide value [mmol O ₂ /kg fat]	Conjugated dienes [-]	Linoleic acid [g/100 g fat]	Linolenic acid [g/100 g fat]
MinF–MinO	175 \pm 23 ^b	56.8 \pm 4.6 ^b	21.7 \pm 2.3 ^a	0.35 \pm 0.05 ^{a,b}
MinF–MaxO	238 \pm 27 ^a	69.1 \pm 2.7 ^a	18.5 \pm 1.2 ^a	0.27 \pm 0.04 ^{a,b}
MaxF–MinO	225 \pm 25 ^{a,b}	61.9 \pm 1.1 ^{a,b}	22.4 \pm 5.0 ^a	0.38 \pm 0.05 ^a
MaxF–MaxO	190 \pm 5 ^{a,b}	57.9 \pm 2.1 ^b	20.4 \pm 1.2 ^a	0.25 \pm 0.05 ^b

Different letters within a column indicate significantly different means ($p < 0.05$).

Analysis of the peroxide value and absorbance of conjugated dienes are appropriate tools to determine primary oxidation markers. **Table 2** depicts the outcomes with the four yellow pea batters

using both techniques. At closer inspection, it became apparent that the two approaches described a similar trend: while *MaxF–MinO* and *MaxF–MaxO* could not be discriminated from the standard *MinF–MinO*, a significant accumulation of primary markers was measurable in the case of *MinF–MaxO*. This finding indicated intensified lipid oxidation in the batter produced using a longer beating time after oil addition, which contrasted with the results of the VOC analysis. It was therefore assumed that lipid oxidation occurred during the initial stage and the beating time was insufficiently long to convert the hydroperoxides thus generated into volatiles. Conversely, this appeared to be the case for the batter with maximum mixing duration (*MaxF–MaxO*) as it was characterized by a significantly higher quantity of VOCs (**Table 1**) and a reduced concentration of hydroperoxides (**Table 2**), thus implying the successful transformation of primary into secondary markers. The greater susceptibility of polyunsaturated fatty acids to oxidative degradation during prolonged flour and oil mixing was likewise noticeable from the significantly lower content of linolenic acid in *MaxF–MaxO* compared to the other products (**Table 2**).

3.4 Correlation between mixing time and local distribution of reactive ingredients

Cake batter is considered as a liquid foam that consists of a continuous aqueous phase containing proteins in which lipids are emulsified and both gas cells and starch granules are dispersed. In order to prevent foam collapse, the stabilization of these air bubbles is achieved by surface-active proteins and lipids, which are naturally present in the ingredients; i.e. egg, flour and oil (Godefroidt, Ooms, Pareyt, Brijs, & Delcour, 2019). The microstructure of batters is determined by the beating protocol applied. Modifications to mixing might therefore be accompanied by changes to the distribution of air cells, water-soluble proteins (e.g. enzymes) and lipid droplets, which could affect their availability to participate in lipid oxidation. In addition, the duration of beating is typically correlated with the amount of air, and thus oxygen, entrapped in the continuous phase of the batter (Bousquière, Michon, & Bonazzi, 2017; Monnet, Jeuffroy, Villemejane, & Michon, 2020), thereby potentially impacting the degree of oxidative fatty acid degradation. For this reason, it was of particular importance to investigate changes in the structural properties of the batters as a function of mixing times after the incorporation of flour and oil.

Fig. 2 shows CLSM images of the four batters using red and green dyes to stain lipids and proteins, respectively. In the case of *MinF–MinO*, a non-homogenous distribution of proteins and lipids could be seen. Moreover, dark disks of various diameters were observable, representing the

incorporated air bubbles that caused a batter density of 0.62 g/cm³ (**Table 3**). This large and uneven size distribution of gas cells might indicate only partial stabilization by surface-active compounds such as lipids, which appeared to be incompletely emulsified as visible from the occurrence of large lipid droplets.

A similar image was captured when increasing the mixing time after flour addition from 30 s to 3 min (*MaxF–MinO*). Compared to the standard batter, however, *MaxF–MinO* was characterized by a significantly higher batter density of 0.71 g/cm³ (**Table 3**), indicating a loss of previously entrapped air. According to Bousquières *et al.* (2017), this might be explained by the mechanical disruption of air cells by the solid flour particles.

On the other hand, the sole prolongation of mixing after oil incorporation by 3 min perceptibly modified the microstructure of the corresponding *MinF–MaxO* batter (**Fig. 2**). Despite a total batter preparation time similar to that of *MaxF–MinO*, a significantly higher density in *MinF–MaxO* was determined (0.77 g/cm³) (**Table 3**). This indicated a greater loss of air, which was presumably due to the degassing effect of the sunflower oil. This phenomenon had likewise been reported by Monnet *et al.* (2020) who studied the effects of the order of ingredient incorporation on the properties of cake batters. At the same time, this beating protocol appears to have led to a reduction in the size not only of air cells but also of fat droplets, which was accompanied by an enlargement of the reaction interface, promoting lipid oxidation. As a second consequence, an improved emulsification of lipids as well as a more homogenous distribution of lipids, proteins and air bubbles was achieved, which increased the likelihood of contact between LOX and its substrates. These structural properties of *MinF–MaxO* therefore provided a substantiated explanation for the elevated levels of primary lipid oxidation markers (i.e. conjugated dienes and hydroperoxides) compared to *MinF–MinO* and *MaxF–MinO* (**Section 3.3**).

As soon as the batter beating times after flour and oil addition were both extended, synergistic effects became apparent. From a structural perspective, no clear distinction between *MaxF–MaxO* and *MinF–MaxO* could be made as the distribution of macromolecules and air cells, as well as batter density, were very similar (**Table 3, Fig. 2**). However, significantly higher concentrations of secondary oxidation markers (i.e. volatile compounds) were identified in this product (**Table 2**). In addition to the surface enlargement effects, this could also be attributed to a significantly longer mixing time, which favored the enzymatically catalyzed breakdown of fatty acids. Interestingly, the increase in batter

density did not affect fatty acid degradation, although it reflected a reduced amount of air, and thus of incorporated oxygen, which was required for lipid oxidation. This led to the conclusion that solubilized oxygen was sufficient to trigger oxidation, which was in line with the results reported by Krause *et al.* (2021a).

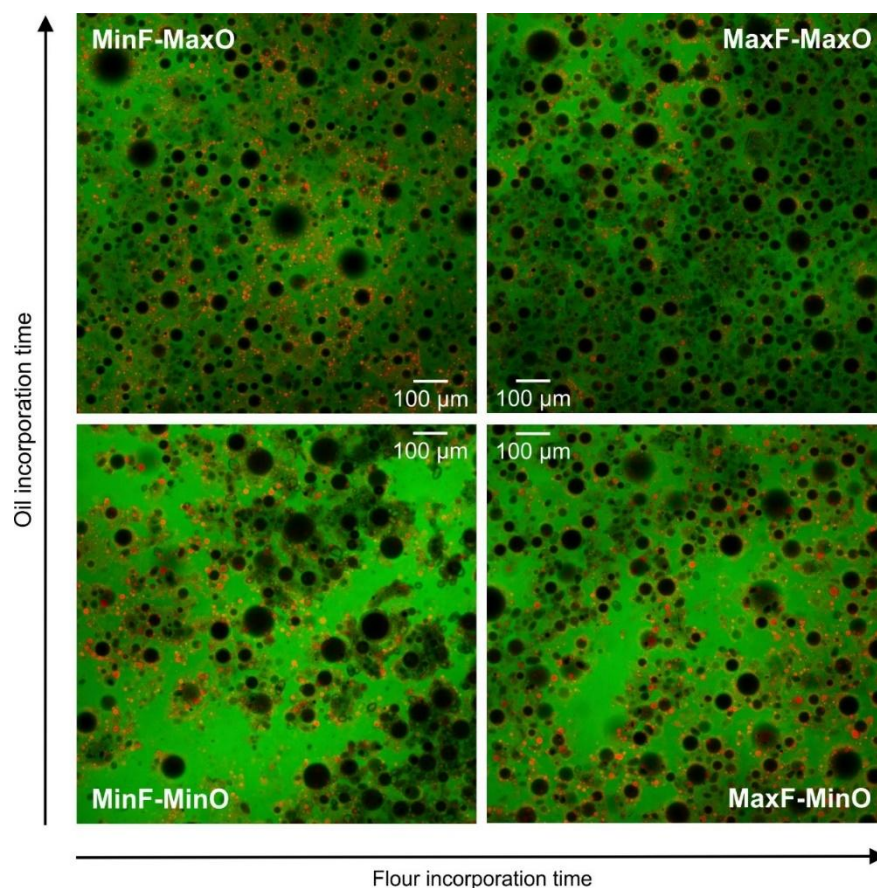


Fig. 2. Microstructure of yellow pea batters prepared using different mixing times after the addition of flour and oil. Proteins are stained in green, lipids in red. Dark circles represent air bubbles. Magnification: 10 \times , scale bar: 100 μ m.

Table 3. Density and pH value of yellow pea batters prepared using different mixing times after the addition of flour and oil (mean \pm SD, $n = 3$).

Batter	Density [g/cm ³]	pH value [-]
MinF–MinO	0.62 \pm 0.01 ^c	7.22 \pm 0.03 ^a
MinF–MaxO	0.77 \pm 0.02 ^a	7.23 \pm 0.00 ^a

MaxF–MinO	0.71 ± 0.01^b	7.24 ± 0.02^a
MaxF–MaxO	0.78 ± 0.02^a	7.21 ± 0.01^a

426 Different letters within a column indicate significantly different means ($p < 0.05$).

4 Conclusion

Lipid oxidation played a crucial role in the development of sponge cake batters containing yellow pea flour, which was mainly attributable to the activity of lipoxygenase present in the flour. The batter we produced was characterized by a complex mixture of volatile organic compounds, essentially composed of alcohols, followed by ketones and aldehydes. Most of these molecules are known to originate from 13- and 10-hydroperoxides of linoleic and linolenic acid, thus suggesting the predominant activity of 13- and 10-lipoxygenases, preferably introducing dioxygen at positions 13 or 10 of the fatty acid carbon chain.

Interestingly, the intensity of fatty acid degradation appeared to be influenced by the ingredient mixing process applied, owing to a combined impact on the local distribution of reactive ingredients and the exposure time of lipoxygenase to its substrates. When the batter beating time was increased after the addition of flour from 30 sec initially to 3 min, neither the quantities of hydroperoxides nor those of volatile compounds varied significantly; this indicated that the longer contact time between lipoxygenase and fatty acids from egg and flour was insufficient to intensify lipid oxidation. This could be assigned to the uneven partitioning and high proportion of large air bubbles and lipid droplets in the batter, which limited their probability of contact. In contrast, prolonging batter beating after the addition of oil by a similar duration of 3 min led to a significant accumulation of hydroperoxides, which implied an elevated degree of lipid oxidation. This supposedly resulted not only from improved batter homogeneity but also from a reduced size, thus enlarging the reaction surface of both lipid droplets and air bubbles, promoting chemical reactivity. Notwithstanding this, the higher potential for fatty acid breakdown after a modified oil mixing time was not reflected in a higher level of volatile compounds. This signified that during the selected extension to beating, the intermediate lipid oxidation markers had not yet been subjected to subsequent cleavage. However, this phenomenon was visible as soon as both mixing times after oil and flour incorporation were extended sequentially by a total of 5.5 min. While the corresponding batter was significantly enriched in volatile compounds, a lower level of hydroperoxides was detected, thus implying the successful transformation of primary into secondary lipid oxidation markers. Because the microstructure of this batter was similar to that obtained after prolonged oil mixing time, the greater potential to generate volatiles was also assigned to a longer contact between the enzyme and its substrates.

456 Based on these findings, it was concluded that by varying the mixing process parameters, a
457 significant effect can be achieved on the degree of lipid oxidation during the development of legume-
458 based products. This valuable knowledge of the opportunities to control the generation of volatiles with
459 potential effects on the sensory attributes of the final food will help to exploit the potential of less
460 refined legume ingredients, such as whole pea flour, and thus to contribute to the design of more
461 sustainable foods.
462

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580

581 **Key references:**

- 582 Krause *et al.* (2021a) This is our previous work on the ability of pulse flours to generate volatile
583 compounds during sponge cake development, which raised new
584 questions about the elevated reactivity of pea flour during food
585 processing to which we wanted to answer with the present study. It lays the
586 foundation for the different methods applied in this study.
- 587 Maire *et al.* (2013) This work supports the strategy to link VOC analysis with the
588 determination of primary oxidation markers, namely conjugated dienes and
589 hydroperoxides, to describe the oxidation status of samples and progress
590 during sponge cake development.
- 591 Monnet *et al.* (2020) This work demonstrates the role of mixing in the structuration of batters
592 and cakes and discusses the changes when substituting wheat flour by
593 pea flour. It made us reflect about the importance of ingredient
594 functionality and raised the question about their availability to participate in reactions.
- 595 Fischer *et al.* (2020) This is an excellent review that highlights different aspects of pea
596 ingredients in comparison to soy, including a section about LOX activity,
597 its different isoenzymes, regiospecificities and pH optima.
- 598 Belitz *et al.* (2009) This book provides in-depth information about the chemistry of food and
599 the chemical reactions taking place during food processing, storage and
600 handling. The principle of lipid oxidation is discussed as well as the
601 decomposition of hydroperoxides into volatile compounds.