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Federal Research Centre for Nutrition, Institute for Food Chemistry, Karlsruhe (Fed. Rep. of Germany)

Enzymatic degradation of polar lipids in deep-frozen parsley

R. Duden and *A. Fricker*)*

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1 Introduction

Vegetable tissues contain beside a great many different lipids also enzyme systems linked to these lipids by metabolic functions. Sastry and Kates (1) found "galactolipases" in bean leaves, for instance, and Galliard demonstrated "lipid acylhydrolases" in potatoes (2) and cucumbers (3).

If the tissue is destroyed mechanically or by freezing, these regulating mechanisms of the intact tissue stop functioning; uncontrolled degradation reactions take place and become responsible for the loss of certain lipids within a short time.

These degradation reactions are closely related to the formation of aroma compounds, as has been discussed in great detail by Galliard for cucumbers (3), by de Lumen et al. for beans (4) and by Tressl for apples (5).

The authors assume a sequence of interdependent enzymatic reactions to take place by which free fatty acids (FFA) are split off hydrolytically and are subsequently transformed by lipoxygenase into hydroperoxy acids, which are transformed enzymatically into aroma compounds such as hexanal and hexenal, for instance. That lipoxygenase does not at all – or only at very low rates – attack fatty acids in the form of glycerol esters is assumed also by other authors (see, for instance, 6, 7, 8, 9; different from these: 10).

Processes of lipid degradation have been made responsible also for the formation of off-flavour compounds during frozen storage. Lee and Wagenknecht have already regarded the formation of undesirable taste components in deep-frozen peas (11) and in other vegetables (12) to be due to the formation of free fatty acids and their oxidation by lipoxygenase. Bengtsson and Bosund (6) studied the kinetics of the formation of free fatty acids in frozen peas and found a considerable hydrolysis rate at temperatures as low as –18 °C. Whitfield and Shipton (13) showed different aldehydes in non-blanched peas stored for 2 years at –18 °C; of these aldehydes, acetaldehyd formed the greatest part. In model tests on linolenic acid/lipoxygenase at –5, –10 and –15 °C Fennema and Sung (14) used the increase of the UV-absorption at 234 nm as a measure for the enzyme effect.

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Purr (15) also attributed the fat spoilage in water-deficient foods to lipolytic-lipoxidative coupling. He found such a reaction sequence also in model mixtures of lecithin, phospholipase A + B and soy lipoxygenase. Galliard (16), too, assumed on the basis of studies in potato tubers that a relation exists between combined hydrolase-lipoxygenase activities and the development of off-flavour components. Rhee and Watts (17) found, on the other hand, that the degree of lipid oxidation in non-blanched peas stored for about 1 year at −10 °C was too low to cause rancidity. Sensory analysis of the non-blanched peas stored in frozen condition also indicated that rancidity was not the main cause for the quality loss in the product. The authors assume therefore that other enzymatic raction pathways "involving anaerobiosis and fermentative changes" might be responsible for the formation of off-flavour.

In the discussions of the lipid degradation processes and their possible significance for the formation of aroma and off-flavour, only little attention if any has been paid to a third enzymatic reaction type, namely the acyl transfer, as has been described by Heinz (18, 19) in great detail. The knowledge of the course of transacylation reactions, however, may possibly be of some importance for the evaluation and control of complex enzymatic processes influencing the storage behaviour of green vegetable tissue.

Heinz demonstrated the formation of 6-acyl-monogalactosyl-diglycerides (AGDG) and – to a much lower extent – also of other galactosyl glycerides in spinach leaves. In own studies (not published) we observed the formation of AGDG also in homogenates of leaves of other vegetables (celery, green cabbage, beans). The degradation rate of monogalactosyl-diglycerides (MGDG) and of digalactosyl diglycerides (DGDG), as well as the decrease of the total ester concentration and the formation of AGDG in homogenates of parsley leaves have been studied in more detail (20).

As far as we know there is no information available on the formation of acylated galactolipids in frozen condition; this has been reason for us to study the lipid degradation under subfreezing conditions as a function of storage time and storage temperature.

Parsley seemed to be an interesting vegetable for our investigations, because it is not blanched before freezing so that the enzyme systems concerned are still intact also under conditions prevailing in practice.

2 Material and methods

2.1 Preparation of samples and storage

Of parsley, which had been obtained from the local commercial market, larger stems were removed; the material was washed, packaged in plastic pouches, frozen at a high freezing rate at −50 °C in flat layers and stored subsequently at −12; −18; −24; −32 and −50 °C. To stop the degradation processes after the intended storage times, the samples were placed into a freezer cabinet showing a temperature of −70 °C where they remained until further treatment. Samples to be blanched were immersed for 1 min in boiling water.

2.2 Extraction of the lipids

After storage, all samples were freeze-dried; 85 mg of each of the individual samples were extracted twice for 20 min at room temperature by using 5 ml

chloroform/methanol 2:1 for each extraction. (Since we were interested only in obtaining relative values, further extraction steps were not necessary; by extracting each sample twice, about 95 % of the individual substances were recovered.)

The solutions which had been cleared by centrifugation were dried under nitrogen, dissolved in 0.5 ml chloroform and stored in vials of 1 ml volume closed with screw top and teflon-coated seal. The tightness of the cover was checked regularly by weighing the vials.

2.3 Chromatography

The lipid extracts were chromatographed on 10 × 10 cm thin- layer plates (Merck, No. 5631, "Nano-DC"), with chloroform/methanol/water/glacial acetic acid 75 : 25 : 4 : 1 being used for the determination of MGDG and DGDG, and ether/ benzene/ethanol/glacial acetic acid 40 : 50 : 2 : 0.3 for AGDG and free fatty acids. The spots were made visible by spraying the plates with 50 % sulfuric acid and heating for 5 min at 180 °C.

2.4 Quantitative evaluation

The galactolipids (MGDG, DGDG, AGDG) were quantitatively evaluated by using remission photometry (ZEISS chromatogram – spectrophotometer). The peak areas F of the densitograms and the respective substance quantities M were related to each other in the case of MGDG by

$$F^2_{MGDG} = K_1 \quad M_{MGDG}$$

and in the case of DGDG by

$$F^{3/2}_{DGDG} = K_2 \quad M_{DGDG}$$

(K_1 and K_2 are constants).

These functions – expressed in % of the – 50 °C (= zero time) values – were used as "relative substance quantities" for the graphical representation of the MGDG and DGDG degradation.

The 6-acyl-monogalactosyl diglycerides the identity of which had been verified by cochromatography with authentic material were chromatographed together with different quantities of hydrated AGDG as external standard. In the case of the AGDG the peak areas F were proportional to the substance quantities.

The quantities of free fatty acids formed were estimated after chromatographic separation by direct measurement of the spot areas (21). For preparing the plates it was found necessary to modify the method described by using a silica-gel suspension adjusted to pH 7.2; with more acidic layers, diffuse spots were obtained which could not be evaluated. Mobile phase: ether /benzene / ethanol / glacial acetic acid 40 : 50 : 2 : 0.3.

2.5 Sensory analyses

The parsley was presented for sensory analysis in three different preparations:

1. The leaves were not minced and tasted immediately after thawing at adequate temperature.
2. The material was finely minced in frozen condition and analyzed sensorily in this form after thawing at adequate temperature.
3. Samples of 20 g finely minced parsley were shortly before tasting given into 0.5 l of a white flour sauce of neutral taste, which was kept in a boiling water bath (light sauce instant, Maggi); the preparation was tasted after cooling down to 50–60 °C.

If the parsley was presented to the tasters in raw condition (1 and 2), the sensory analysis was difficult, because the bitter-sharp taste of the samples masked the remaining taste attributes; having tasted the first sample, the tasters were no longer

able to feel any differentiated perceptions. Added to the sauce, the parsley lost its bitter note, however, and its aroma could fully develop.

We therefore confined our tests to preparations in light sauce. The evaluations were based on the Karlsruhe Evaluation Scheme (22) and concentrated on the taste only. Good to very good properties received 7–9 points, average qualities 4–6 points; less than 3 points indicated that the material was no longer suitable for consumption.

3 Results

3.1 Lipid degradation

Figure 1 shows a thin-layer chromatogram of the extracts of parsley samples stored in frozen condition over different periods. A comparison of the −12 °C and −18 °C series of nonblanched and blanched samples – the latter representing approximately the lipid composition of the initial material – shows that profound changes took place already after short storage periods.

After 2 weeks at −18 °C the lecithin was completely degraded, a process requiring much less time at −12 °C. As the chromatograms show, mono-

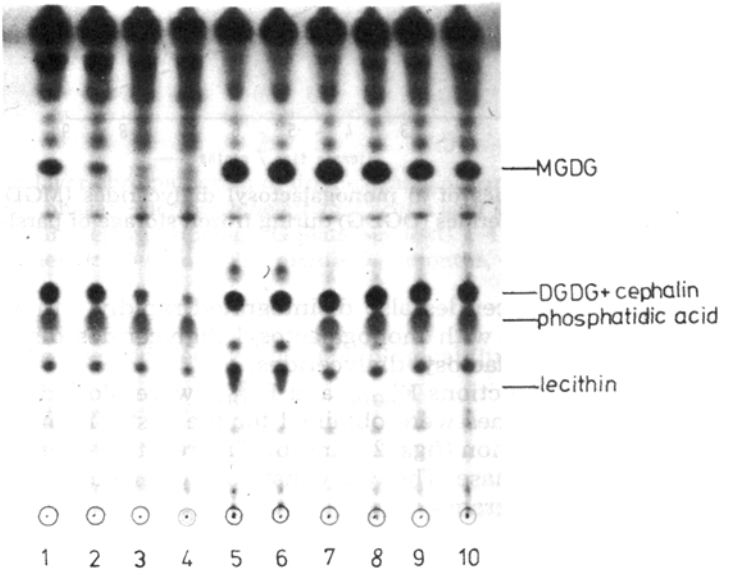

Fig. 1. Thin-layer chromatogram of lipid extracts from parsley stored in frozen condition. Mobile phase: chloroform/methanol/water/glacial acetic acid 75 : 25 : 4 : 1; saturated chamber. 2 µl per spot were applied.

Non-blanched samples:
1: −12 °C, 0.5 month
2: −12 °C, 1 month
3: −12 °C, 3 months
4: −12 °C, 6 months
7: −18 °C, 0.5 month
8: −18 °C, 1 month
9: −18 °C, 3 months
10: −18 °C, 6 months

Blanched controls:
5: −18 °C, 3 months
6: −18 °C, 6 months

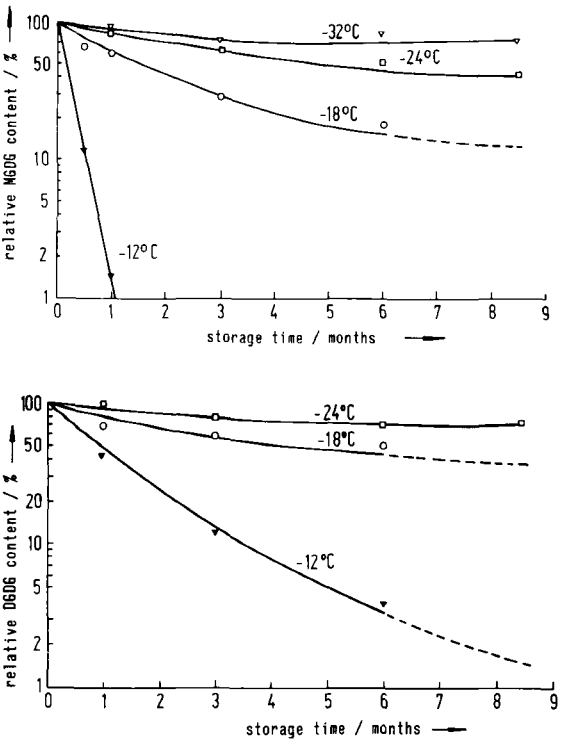

Fig. 2. Degradation kinetics of a) monogalactosyl diglycerides (MGDG) and b) digalactosyl diglycerides (DGDG) during frozen storage of parsley.

and digalactosyl diglycerides also disintegrated rapidly – though much slower than lecithin –, with monogalactosyl diglycerides disintegrating more rapidly than digalactosyl diglycerides.

If the peak area functions F^2_{MGDG} and $F^{3/2}_{DGDG}$ were plotted against the time, nearly straight lines were obtained for the first 3 months in semilogarithmic representation (figs. 2 a and b). The reactions hence are first-order in their initial phase. The rate constants compiled in table 1 were derived from these diagrams.

Table 1. Constants of the degradation rate of MGDG and DGDG (mon^{-1}).

°C	– 32	– 24	– 18	– 12
MGDG	0.036	0.073	0.18	1.86
DGDG	–	0.024	0.084	0.29

It becomes obvious that the enzymatic reactions do not come to a complete standstill even at – 32 °C. Whether the standstill was complete at – 50 °C or whether some reactions still take place to a very little extent

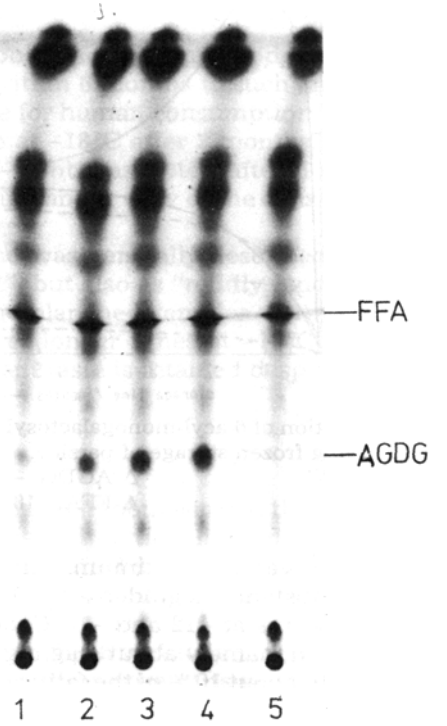

Fig. 3. Thin-layer chromatogram of lipid extracts of parsley stored in frozen condition; Mobile phase: ether/benzene/ethanol/acetic acid 40 : 50 : 2 : 0.3 (unsaturated chamber). Self-prepared silica-gel G plate (see text). 1–4 non-blanched; 5: blanched. storage times: 1: 0.5 month, 2: 1 month, 3: 3 months, 4: 6 months, 5: 3 months.

could not be decided because of the biological fluctuations within the test material.

3.2 Reaction products

In the non-blanched samples in which the lecithin disappeared completely (−12 °C; −18 °C; −24 °C) considerable quantities of phosphatidic acid were detected (fig. 1), a fact indicative of phospholipase D effect. Phosphatidic acid was detected on the chromatograms also after storage at −32 °C and even at −50 °C; however, at these temperatures no relation between time and quantities could be noted, which gives rise to the assumption that the corresponding reactions took place not during storage, but during freeze-drying of the samples.

Figure 3 shows clearly the formation of considerable quantities of 6-acyl-monogalactosyl diglycerides during storage (acyltransferase effect). The quantitative evaluation of the chromatograms is demonstrated in figure 4.

At −12 °C the acylgalactosyl diglycerides formed at the beginning of storage degraded slowly, with a maximum content resulting after about

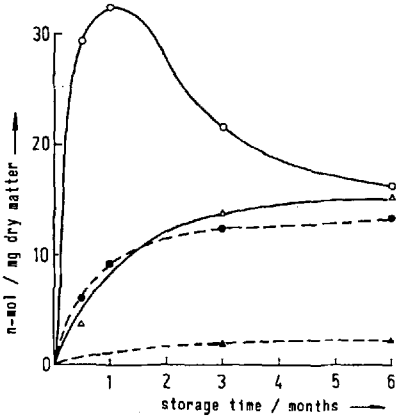

Fig. 4. Kinetics of the formation of 6-acyl-monogalactosyl diglycerides (AGDG) and free fatty acids (FFA) during frozen storage of parsley.
○ AGDG, −12 °C △ AGDG, −18 °C
● FFA, −12 °C ▲ FFA, −18 °C

1 month. At −18 °C there was no maximum, but a slow increase up to 6 months. Whether the substance degraded again later was not examined.

After storage for 6 months at −12 and −18 °C, only small quantities of free fatty acids had formed, namely about 4 µg/mg dry matter at −12 °C, a quantity corresponding to about 10 % of the fatty acids originally bound to the galactosyl glycerides. At −18 °C about 0.5–1 µg/mg dry matter had formed after 6 months.

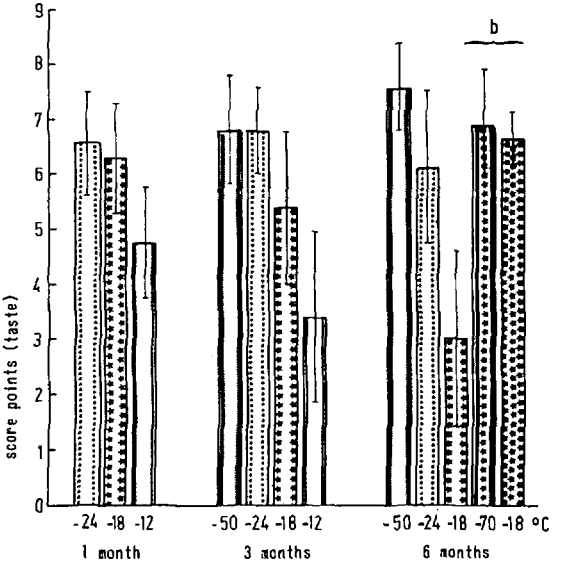

Fig. 5. Sensory evaluation of parsley stored in frozen condition over different storage times. Samples marked by b were blanched – the remaining ones were non-blanched

3.3 Sensory analyses

The taste of the non-blanched samples decreased at −12 °C within 3 months and at −18 °C within 6 months to such an extent that the samples were no longer suitable for human consumption (fig. 5). A distinct quality loss was observed also at −18 °C after 3 months. Even at −24 °C a quality loss of about 1 score point was noted after 6 months – however, this difference was not significant in view of the considerable range of fluctuation of the sensory tests.

The off-flavour trend was generally described as "old", "not typical", "strange", "unpleasant", but also as "mildly rancid", "tasting of fish oil", "like fish", whereas the blanched samples were rated surprisingly high. They can be stored over longer times at −18 °C without distinct quality loss. A fresh and pleasant taste is retained despite blanching.

4 Discussion

The frequently expressed hypothesis that the spoilage of non-blanched frozen vegetables follows hydrolytical-lipoxidative reaction pathways has not been experimentally verified so far. The model tests of Fennema and Sung (14), who demonstrated the formation of hydroperoxidienoic acids out of linolenate between −5 and −15 °C, are little informative in this respect as long as it has not been verified that free fatty acids of the linoleic acid type are released at all in frozen tissue or that intact esterlipids like lecithin or galactolipids can be directly attacked by lipoxygenase. But even in peas, in which free fatty acids form in higher quantities, the spoiling mechanism remains controversial; the thiobarbituric acid tests and the sensory studies of Rhee and Watts (17) speak also in this case against lipoxygenase action as cause of spoilage.

The studies of Whitfield and Shipton (13) mentioned above also suggest rather those spoiling mechanisms, which were assumed also by Rhee and Watts; for 96 % of the carbonyl compounds isolated from non-blanched peas stored for 2 years at −18 °C consisted of acetaldehyd, which is formed neither by autoxidation nor by lipoxygenase-catalized peroxidation of non-saturated fatty acids. It has not been examined whether the remaining 4 % of the carbonyl compounds many of which contributed possibly only little to the off-flavour were sufficient to exceed the sensory threshold or to clearly mask any changes caused by different processes.

Neither can the studies of Purr (15) according to which also phospholipids may be the source of spoiling substances in the sense of a hydrolytic-lipoxidative coupling be transferred to vegetables, because the activity ratio between different enzymes can be very different under the conditions of storage in frozen and dried condition.

Phospholipids in frozen parsley are mainly degraded by phospholipase D; under this reaction phosphatidic acid is formed – fatty acids are not released. The reaction sequence assumed by Purr (15) must hence be excluded at least as main reaction pathway for frozen parsley (and probably also for other frozen leafy tissues). We did not find any further degradation of the phosphatidic acid after 6 months at −18 °C so that free fatty acids are not formed in this way either.

The situation is similar with the galactolipids, which form the greater part of the leaf lipids. The acyl group transfer:

$$2\ \text{MGDG} + \text{DGDG} \xrightarrow{\text{acyl transferase}} 2\,\text{AGDG} + \text{digalactosyl glycerol}$$

obviously has primary importance for the galactolipid degradation under the conditions of frozen storage. Also here, lipoxidative coupling:

$$\text{MGDG} \xrightarrow{\text{lipid acyl hydrolase}} 2\ \text{FFA} + \text{galactosyl glycerin}$$

$$\text{FFA} \xrightarrow{\text{lipoxygenase}} \text{hydroperoxidienoic acid} \downarrow \text{off-flavour components}$$

may at most play a minor role for spoiling processes.

The question whether the product quantities resulting from the peroxidation of free fatty acids – split off from galactolipids and/or phospholipids by acylhydrolase activities – is still being investigated.

As far as practice is concerned, our sensory analyses have shown that −18 °C as storage temperature are not sufficient to store non-blanched parsley for longer than 1–2 months. −24 °C are sufficient to preserve adequate quality for up to 1 year. In lack of the technical possibilities to realize these conditions, short blanching is recommended.

Summary

Under the frozen storage at usual storage temperatures of leafy tissues not pretreated by heat, enzymatic lipid degradation reactions take place, which lead already after a few weeks to a considerable or complete loss of the native polar lipids. These degradation processes being accompanied by a deterioration of the flavour have been studied in greater detail in parsley leaves.

Among the reaction products we found large amounts of 6-acylmonogalactosyl diglycerides (formed from monogalactosyl diglycerides by enzymatic transacylation) and phosphatidic acid (formed from phospholipids through phospholipase-D action). The generally assumed reaction sequence: formation of free fatty acids by acyl hydrolases followed by hydroperoxidation through lipoxygenase and degradation of the hydroperoxides into off-flavour compounds may hence take place, if at all, only to a limited extent.

Considerable phospholipase D as well as minor acyl transferase activities are still detected at −24 °C, whereas at −32 °C the lipid loss is very low.

Deterioration processes can be avoided by blanching, a treatment not leading to any substantial quality loss.

Zusammenfassung

Während der Gefrierlagerung von thermisch nicht behandelten Blattgeweben laufen bei praxisüblichen Lagerungstemperaturen enzymatische Lipidabbaureaktionen ab, die schon nach wenigen Wochen zu einem weitgehenden oder vollständigen Verlust der nativen polaren Lipide führen. Diese von einer Geschmacksverschlechterung begleiteten Prozesse wurden am Beispiel von Petersilieblättern näher untersucht.

Unter den Reaktionsprodukten befanden sich beträchtliche Mengen von 6-Acylmonogalaktosyldiglyceriden – entstanden durch Transacylierung aus Monogalaktosyldiglyceriden – sowie von Phosphatidsäure, deren Bildung durch Einwirkung von Phospholipase D auf Phospholipide zu erklären ist. Die vielfach angenommene

Reaktionsfolge: Spaltung der Lipide durch Acylhydrolasen, Hydroperoxidation der gebildeten freien Fettsäuren durch Lipoxigenase und Zerfall der Hydroperoxide in Off-flavour-Komponenten spielt möglicherweise nur eine untergeordnete Rolle.

Die Phospholipase D ist selbst bei −24 °C noch sehr wirksam. Acyltransferasewirkungen sind in geringerem Umfang ebenfalls noch bei dieser Temperatur erkennbar. Bei −32 °C werden die Lipide nur noch in sehr geringem Umfang angegriffen.

Die Verderbprozesse können durch Blanchieren unterbunden werden, ohne daß die Petersilie dadurch eine wesentliche Qualitätseinbuße erleidet.

Key words: galactolipids, phospholipids, frozen storage, parsley

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