The Royal Veterinary and Agricultural University

Formation of Aroma Compounds in Leek during Processing and Storage

- with Emphasis on the Enzymes Lipoxygenase, Hydroperoxide Lyase, Alcohol Dehydrogenase, and Alliinase

Ph.D. Thesis by Ghita Studsgaard Nielsen

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Department of Food Science
The Royal Veterinary and Agricultural University
Rolighedsvej 30, 1958 Frederiksberg C., Denmark

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1. Preface

The leeks used for my experiments were kindly donated firstly by Danisco Foods, and then by the Belgian company Ardo that bought Danisco Foods. I'm very appreciative for this, as it gave me the opportunity to harvest the plant material right before processing. Thank you Peder Krogsgaard for keeping me informed of appropriate harvest time and for guiding me through the roads of Funen to find the right fields of leeks. Also I would like to thank the Belgian headquarters of Ardo in Ardooie, Belgium for inviting me and Leif Poll on a very interesting tour in their plant of frozen leek slice production.

Peter Thane, Gulerodsgruppen A/S, led me have my "own" part of one of the leek fields in Lammefjorden, and for this I'm very thankful as it gave me the valuable opportunity of using the same variety grown at uniform conditions for my many optimizing experiments.

The experimental work was performed in the laboratories of the section of Plant Food Science at the Department of Food Science, The Royal Agricultural and Veterinary University, Copenhagen, Denmark. Over the years a number of laboratory technicians and students have taken part in my research work, and I would like to thank Sanne Petersen, Oddvá Nielsen, Peter Houghton Larsen, Linda Thullesen, Karina A. Topolianaki Fife, Selda Rakipovska, and Mehdi D. Farahani for the help. Also I would like to thank the whole section of Plant Food Science, and especially my office mate Camilla Varming, for the pleasant working atmosphere.

I'm grateful to my co-supervisor Lone Melchior Larsen for always having interesting and relevant comments to my work and thanks for still being involved in discussions all through to the end, even though you retired in the middle of the project. To Leif Poll, my head supervisor, thanks for always having the time to discuss things, for supervising me through the project and the process of writing the thesis, and for being a very good friend.

Last, but not least, I would like to thank my family, my husband Niels and my children Natascha and Nicklas for showing understanding and patience with me especially in the last part of the project, and my parents, Karin and Jørgen, for being there all the time for the children.

March 2004

Ghita Studsgaard Nielsen

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2. Summary

The objective of the present Ph.D. thesis was to investigate the formation of aroma compounds and the activity of the enzymes alliinase, lipoxygenase (LOX), hydroperoxide lyase (HPL), and alcohol dehydrogenase (ADH) in leek slices during processing and frozen storage. The thesis consists of a theoretical part describing the present relevant knowledge in the field of aroma formation in leek and other *Allium* species as affected by processing, and an experimental part, which comprises the publications the project gave rise to so far. All through the theoretical part these publications are cited together with other publications, but with clear indication.

The theoretical part describes the formation of aroma compounds in fresh leek. The intact plant does not possess any aroma but as the tissue is damaged by cleaning and cutting, formation of the species characteristic sulfur compounds and the common aldehydes and degradation products of these compounds starts. Sulfur compounds are important for the aroma of leek while aldehydes will appear as off-flavor when present in larger concentrations. The most important enzymes (alliinase, LOX, HPL, and ADH) responsible for the aroma formation in leek are characterized, and the factors affecting the aroma composition by processing are outlined.

The experimental part is reported in seven publications. Five of these publications are concerned with aroma composition of leek slices during frozen storage as affected by processing parameters. The effect of slice thickness, blanching, storage atmosphere and storage time on the enzyme activity and aroma formation in frozen leek slices was investigated in various storage experiments each of the duration of one year. One publication refers to the impact of blanching on the aroma profile of fresh leek slices, and one publication investigates the formation of volatile compounds from crude leek enzyme extract and unsaturated fatty acids.

Pronounced alterations of the aroma composition occurred during storage of frozen leek slices, and this was influenced by all of the parameters mentioned above. Slice thickness had an effectual impact on the formation of sulfur compounds which was largest in the thin slices. Blanching prior to frozen storage prevented formation of aldehydes (off-flavor) during the storage which indicates that these compounds originate mainly from enzymatic processes rather than autoxidation. The concentration of sulfur compounds diminished most in the unblanched samples during frozen storage.

Packaging in 100% nitrogen had a positive influence on the keeping of the initial concentration of sulfur compounds, and did to some degree prevent formation of aldehydes during frozen storage. A combination of blanching and 100% nitrogen in the package atmosphere was shown to almost maintain the initial aroma profile of the leek slices.

Enzyme activity decreased during frozen storage; alliinase, HPL and ADH activity was diminutive after one year, and LOX activity was reduced to ~25% of the initial. The aroma

analyses demonstrated that production of aldehydes was possible on the basis of the reduced enzyme activity. LOX was the most heat labile enzyme and HPL was the most heat stabile enzyme.

The most important odor active compounds in fresh leek were dipropyl disulfide, methyl 1-propenyl disulfide, pentanal, decanal and propyl 1-propenyl disulfide in order of priority. This alters during frozen storage of unblanched leek slices to pentanal, decanal, 2,5-dimethyl furan, an unknown compound and dipropyl disulfide. Blanching to some degree maintains the order of odor active compounds found in the fresh leek slices but the intensity is diminished.

3. Sammendrag

Formålet med den nærværende Ph.D. afhandling var at undersøge dannelse af aromastoffer og aktivitet af enzymerne alliinase, lipoxygenase (LOX), hydroperoxide lyase (HPL) og alkohol dehydrogenase (ADH) i porreskiver under forarbejdning og frostlagring. Afhandlingen består af en teoretisk del, der beskriver allerede eksisterende viden om forarbejdningens påvirkning af aromadannelse i porrer og andre *Allium* arter, og en eksperimentel del, der består af de artikler projektet har givet anledning til indtil nu. Gennem den teoretiske del er disse artikler citeret sammen med andre artikler, men med tydelig indikering heraf.

Den teoretiske del beskriver dannelse af aromastoffer i friske porrer. Den intakte plante udsender ingen aroma, men når vævet ødelægges ved rensning og opskæring, starter dannelsen af de karakteristiske svovlforbindelser og de almindeligt forekommende aldehyder og nedbrydningsprodukter heraf. Svovlforbindelser er vigtige for porrearomaen, mens aldehyder i højere koncentrationer vil optræde som off-flavor. De vigtigste enzymer i denne aromadannelse (alliinase, LOX, HPL og ADH) bliver beskrevet, og faktorer i forarbejdningen, der kan påvirke aroma sammensætningen, gennemgås.

Den eksperimentelle del er afrapporteret i 7 artikler. 5 af disse omhandler produktionsmetoders indflydelse på aroma sammensætning i porreskiver under frostlagring. Virkningen af snittetykkelse, blanchering, pakkeatmosfære og lagringstid på enzym aktivitet og aromadannelse i frosne porreskiver blev undersøgt gennem lagringsperioder på 1 år. En artikel undersøger blancherings indflydelse på aroma profilen i friske porrer og en artikel omhandler dannelse af flygtige forbindelser ud fra råekstrakt af porreenzym og umættede fedtsyrer.

Der skete store ændringer i aromasammensætningen under frostlagring af porreskiver, og dette var påvirket af alle de ovenfor nævnte parametre. Snittetykkelse havde stor effekt på dannelsen af svovlforbindelser, der var størst ved mindst snittetykkelse. Blanchering inden frostlagring forhindrede dannelse af aldehyder (off-flavor) under lagringen, hvilket indikerer at disse forbindelser hovedsagligt stammer fra enzymatiske reaktioner og ikke autoxidation. Koncentrationen af svovlforbindelser blev mest reduceret i de ikke-blancherede prøver under frostlagring.

Pakning i 100 % nitrogen havde en positiv indflydelse på opretholdelse af den oprindelige koncentration af svovlforbindelser, og dannelse af aldehyder blev i nogen grad forhindret under frostlagring. En kombination af blanchering og pakning i 100 % nitrogen viste sig at bibeholde den oprindelige aroma profil i porreskiverne bedst.

Enzym aktiviteten faldt under frostlagring. Alliinase, HPL og ADH aktiviteten var minimal efter 1 år, mens LOX aktiviteten blev reduceret til ca. 25 % af den oprindelige aktivitet. Aroma

analyserne viste, at produktion af aldehyder var mulig på grundlag af den reducerede enzym aktivitet.

De vigtigste stoffer i porrearoma er dipropyl disulfide, methyl 1-propenyl disulfide, pentanal, decanal og propyl 1-propenyl disulfide i prioriteret rækkefølge. Efter en frostlagring på 1 år er dette ændret til pentanal, decanal, 2,5-dimethyl furan, et ikke-identificeret stof og dipropyl disulfide. Blanchering af porreskiverne gør, at rækkefølgen for de friske porrer stort set er bibehold efter 1 års lagring, men intensiteten af de enkelte stoffer er mindre.

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4. List of Abbreviations

GC-O	gas chromatography olfactometry
NIF	nasal impact factor
SNIF	surface of nasal impact factor
GC-MS	gas chromatography mass spectrometry
HPLC	high performance liquid chromatography
ACSO	*S*-alk(en)yl-L-cysteine sulfoxide
MCSO	(+)-*S*-methyl-L-cysteine sulfoxide
PCSO	(+)-*S*-propyl-L-cysteine sulfoxide
(*E*)-1-PeCSO	(*E*)-(+)-*S*-1-propenyl-L-cysteine sulfoxide
(*Z*)-1-PeCSO	(*Z*)-(+)-*S*-1-propenyl-L-cysteine sulfoxide
2-PeCSO	(+)-*S*-2-propenyl-L-cysteine sulfoxides
ECSO	(+)-*S*-ethyl-L-cysteine sulfoxides
BCSO	(+)-*S*-butyl-L-cysteine sulfoxides
LOX	lipoxygenase
HPL	hydroperoxide lyase
HPD	hydroperoxide dehydrase
ADH	alcohol dehydrogenase
P-5'-P	pyridoxal-5'-phosphate
HPODE	hydroperoxide of linoleic acid
9-HPODE	9-(*S*)-hydroperoxy-10-(*E*)-12-(*Z*)-octadecadienoic acid
13-HPODE	13-(*S*)-hydroperoxy-9-(*Z*)-11-(*E*)-octadecadienoic acid
9-HPOTE	9-(*S*)-hydroperoxy-10-(*E*)-12-(*Z*)-15-(*Z*)-octadecatrienoic acid
13-HPOTE	13-(*S*)-hydroperoxy-9-(*Z*)-11-(*E*)-15-(*Z*)-octadecadienoic acid
Val	valine
Phe	phenylalanine
0 M	fresh leek slices
12 M	frozen leek slices stored for 12 M

5. List of Publications

The thesis is based on the experimental work reported in the following seven publications, referred to in the text by **paper 1-7**.

Paper 1. Nielsen G. S.; Larsen L. M.; Poll L. Formation of aroma compounds and lipoxygenase (EC 1.13.11.12) activity in unblanched leek (*Allium ampeloprasum* Var. *Bulga*) slices during long-term frozen storage. Journal of Agricultural and Food Chemistry, **2003**, *51*, 1970-1976.

Paper 2. Nielsen G. S.; Poll L. Impact of water blanching on the retention and formation of aroma compounds in leeks (*Allium ampeloprasum* Var. *Lancelot*). In: *Flavour research at the dawn of the twenty-first century. Proceedings of the 10th Weurman Flavour Research Symposium*; Eds.: Le Quéré J. L.; Etiévant P. X. Cachan, France: Lavoisier, **2003,** pp 678-681.

Paper 3. Nielsen G. S.; Larsen L. M.; Poll L. Formation of aroma compounds during long-term frozen storage of unblanched leek (*Allium ampeloprasum* Var. *Bulga)* as affected by packaging atmosphere and slice thickness. Journal of Agricultural and Food Chemistry, **2004**, *52*, 1234-1240.

Paper 4. Nielsen G. S.; Poll L. Determination of odor active aroma compounds in freshly cut leek (*Allium ampeloprasum* Var. *Bulga)* and in long-term stored frozen unblanched and blanched leek slices by gas chromatography olfactometry analysis. Journal of Agricultural and Food Chemistry, **2004**, *52*, 1642-1646.

Paper 5. Nielsen G. S.; Larsen L. M.; Poll L. Formation of volatile compounds in model experiments with crude leek (*Allium ampeloprasum* Var. *Lancelot*) enzyme extract and linoleic acid or linolenic acid. Accepted for publication in Journal of Agricultural and Food Chemistry, **2004**.

Paper 6. Nielsen G. S.; Larsen L. M.; Poll L. Impact of blanching and packaging atmosphere on the formation of aroma compounds during long-term frozen storage of leek (*Allium ampeloprasum* Var. *Bulga)* slices. Submitted to Journal of Agricultural and Food Chemistry.

Paper 7. Nielsen G. S.; Poll L. Influence of slice thickness and blanching on formation of aroma compounds during frozen storage of leek (*Allium ampeloprasum* Var. *Bulga*) Slices. In preparation for the 7th Wartburg symposium on flavor chemistry & biology, april **2004**. This work will be presented as a poster and a manuscript in the proceedings.

6. Introduction

Leek, onion, garlic, and other members of the genus *Allium* belong to the family *Alliaceae* in the order *Liliales* (19, 88). *Allium* is a genus of bulbous herbs consisting of about 550 species of which a minor part is important as food plants (19). These plants are characterized by their strong odor, for which sulfur compounds to a large degree are responsible (153). The name *Allium* is probably derived from the Celtic word "all", which means pungent (25). Leek to some degree possesses the pungency that is recognized from onion.

The genus *Allium* consists of several eatable species including onion (*Allium cepa* L.), garlic (*Allium sativum* L.), chives (*Allium schoenoprasum* L.), leek (*Allium ampeloprasum* L.), and a lot of not so widespread species. The onion is one of the major horticultural crops in the world and subject to substantial international trade, and probably for that reason many studies are done on the basis of onion. Compared to onion leek is a minor crop; it is grown worldwide but mainly in the northern Europe. The cultivated garden leek is also commonly referred to as *Allium porrum* (46), and due to plant breeding many varieties exist. The common names (leek, onion, garlic ect.) of the species are principally used in this thesis, and there is no distinguish between varieties within species, although there probably are differences, but this is not investigated in relation to aroma formation and enzyme activity in this thesis.

Although *Alliums* normally are described as bulbous vegetables, leek is a tall, hardy biennial plant with little developed bulb (154) but closely related to garlic and onion (44). The leek is divided in two parts, the green leafy part and the white false stem part, which are both eatable (1). Leaves are flat, broad, and folded sharply lengthwise. Their long bases encircling each other to form an elongated cylinder on which the inner leaf bases become etiolated by lack of light (46). The experimental part of this thesis is performed only on the white part, because this part is used in the commercial production of frozen leek slices.

Plants of the genus *Allium* have been recognized as rich sources of secondary metabolites with interesting biological activities (43). The use of *Allium* species as vegetables, spices, and home remedies against illnesses has a long history, and the antibiotic effects of especially garlic have been known for many years (182). Di-2-propenyl thiosulfinate (commonly known as allicin) is a scavenger of peroxy radicals and is

responsible for an antioxidant activity found in garlic bulbs (194). Although the different species of *Allium* have nutritional and therapeutic values in diets, they are mainly consumed for their flavor and their ability to enhance the flavor of other foods (135).

Many *Allium* species are consumed as foods in widespread areas of the world (53). The *Allium* species are a major source of flavoring in fresh and cooked foods and also in the convenience food industry (40). Leek is sold both as whole for the retail marked and as fresh cut and as frozen slices for catering and retail marked. Storage of whole leek for longer periods is not common practice in Denmark; they are harvested all through the winter as long as the soil is not frozen. This is possible because leek are resistant to cold temperatures, and because they are slow bolting (180). Many of the Danish grown leeks are processed into frozen slices, which can be sold at least a year after harvest and processing. This frozen storage introduces alterations in the aroma composition of the leek slices and this can affect the quality of the product.

The objective of the present thesis was to review the relevant literature on the topic of aroma formation in leek during processing and storage, to investigate the formation of aroma compounds and the activity of the enzymes alliinase, lipoxygenase, hydroperoxide lyase, and alcohol dehydrogenase in leek slices during processing and frozen storage, and to study the correlation between enzyme activity and aroma formation. Furthermore to evaluate the importance of the detected aroma compounds for the aroma profile of leek and by that means estimate the impact of processing methods on the quality of frozen leek slices.

7. Aroma Composition of Leek

The chemical composition of *Allium* plants is characterized by the amount of organic bound sulfur, which is much higher than in most other organisms (19, 182). This is interesting both because these sulfur compounds have a potential antibiotic property, and because they are precursors for volatile compounds that act as very strong and pungent aroma compounds. The aroma composition of *Allium* species has been the subject of many investigations. Most of these are done on onions and garlic, which is not surprising as they are important crops on the world marked, whereas few are done on fresh or stored, processed leeks. The majority of the volatile compounds found in fresh *Allium* species are sulfur compounds but also various carbonyl compounds and some other compounds contribute to the aroma.

7.1. Volatile compounds identified in leek

Although the volatile composition of leek has not given rise to many publications, a total of around 90 compounds have been reported. **Table 7.1** displays all of the reported volatile compounds found in the literature concerning fresh or frozen leek tissue. Some investigations are done on leek oil or with very long isolation procedures (143, 144, 159), but as this most likely introduces alterations of the aroma profile as explained in section 7.3, this information is not included in **Table 7.1.**

Table 7.1. Volatile compounds identified in leek. *(*E*)- and/or (*Z*)- isomers of the 1-propenyl radical. **P1**, **P3**, **P4**, and **P6** = **paper 1**, **3**, **4**, and **6**.

Compound	Fresh leek	Frozen stored leek
	Sulfur compounds	
Dimethyl thiosulfinate	(1, 12, 15, 117)	
Methyl propyl thiosulfinate	(1, 12, 15, 48, 117)	
Methyl propenyl thiosulfinate*	(12, 15, 117)	
Methyl 2-propenyl thiosulfinate	(1, 12)	
Dipropyl thiosulfinate	(1, 12, 15, 48)	
Propyl propenyl thiosulfinate*	(1, 12, 15, 48, 117)	
Propyl 2-propenyl thiosulfinate	(1, 12)	
Dipropenyl thiosulfinate*	(48)	
Methyl propyl thiosulfonate	(48)	
Methyl propenyl thiosulfonate*	(48)	
Propyl propenyl thiosulfonate*	(48)	
Thiopropanal-*S*-oxide	(12, 117)	
Hydrogen sulfide	(1)	
Methanethiol	(1)	
Ethanethiol	(1)	
Propanethiol	(1, 145) **P3**, **P4**, **P6**	(125) **P3, P4, P6**

Compound	Fresh leek	Frozen stored leek
2-Propene-1-thiol		(125)
Dimethyl sulfide	(1)	
Methyl pentyl sulfide		**P1**
Di-2-propenyl sulfide	(1)	
Dimethyl disufide	(145) **P1**, **P3**, **P6**	**P3, P6**
Methyl propyl disulfide	(1, 48) **P1**, **P3**, **P4**, **P6**	(125) **P1, P3, P4, P6**
Methyl propenyl disulfide*	(48, 145) **P1**, **P3**, **P4**, **P6**	(125) **P1, P3, P4, P6**
Methyl 2-propenyl disulfide	(1) **P1**, **P3**, **P4**, **P6**	**P3, P4, P6**
Ethyl 1-methylethyl disulfide	**P1**, **P3**, **P6**	**P3, P6**
Dipropyl disulfide	(1, 145) **P1**, **P3**, **P4**, **P6**	(125) **P1, P3, P4, P6**
Propyl propenyl disulfide*	(48, 145) **P1**, **P3**, **P4**, **P6**	(125) **P1, P3, P4, P6**
Propyl 2-propenyl disulfide	(1) **P3**, **P4**, **P6**	(125) **P3, P4, P6**
Dipropenyl disulfide*	(48)	
Di-2-propenyl disulfide	(1)	
Propyl butyl disulfide		**P6**
Propyl pentyl disulfide		**P6**
Dimethyl trisulfide	(145) **P1**, **P3**, **P4**, **P6**	(125) **P1, P3, P6**
Methyl propyl trisulfide	(145) **P3**	(125) **P3**
Diisopropyl trisulfide	**P1**, **P3**, **P4**, **P6**	**P1, P3, P4, P6**
Dipropyl trisulfide	(145)	(125)
Propyl propenyl trisulfide*	**P1, P3, P4**	(125) **P1, P3, P4**
2,5-Dimethyl thiophene	**P3**, **P6**	**P3, P4, P6**
3,4- or 2,4-dimethyl thiophene	(2) **P1**, **P3**, **P4**, **P6**	**P1, P3, P4, P6**
Dimethyl thiophene	(145)	
3,4-Dimethyl-2,5-dioxo-2,5-dihydrothiophene	(2)	
Propyl thioacetate	**P1**	
3,5-Diethyl-1,2,4-trithiolane	**P1**	(125) **P1**
Zwiebelane	(117)	
	Aldehydes	
Methanal	(1)	
Propanal	(1) **P3**, **P6**	**P3, P6**
Butanal	**P4**, **P6**	**P3, P4, P6**
Pentanal	**P3**, **P4**, **P6**	(125) **P1, P3, P4, P6**
Hexanal	**P1**, **P3**, **P4**, **P6**	(125) **P1, P3, P4, P6**
Heptanal		**P1, P3, P6**
Octanal		**P1, P3, P4, P6**
Nonanal	**P3**, **P4**	**P1, P3, P4, P6**
Decanal	**P4**	**P3, P4, P6**

Compound	Fresh leek	Frozen stored leek
(*E*)-2-Butenal		**P1, P3, P6**
(*E*)-2-Pentenal		**P1, P3, P6**
(*E*)-2-Hexenal	(48) **P1**, **P3**, **P4**, **P6**	**P1, P3, P4, P6**
(*Z*)-3-Hexenal	(48)	
(*E*)-2-Heptenal	**P3**, **P4**, **P6**	**P1, P3, P4, P6**
(*E*)-2-Octenal	**P3**, **P4**	**P1, P3, P4**
(*E*)-2-Nonenal	**P1**	(125) **P1, P6**
(*E*)-2-Decenal		**P1**
2-Methyl-(*E*)-2-butenal	(2)	(125) **P1, P3, P6**
2-Ethyl-(*E*)-2-butenal	**P4**	**P1, P3, P4, P6**
2-Methyl-(*E*)-2-pentenal	(2, 48, 117, 145) **P1**, **P3**, **P4**, **P6**	(125) **P1, P3, P4, P6**
(*E*,*Z*)-2,4-Heptadienal	**P1**, **P3**, **P6**	**P1, P3, P6**
(*E*,*E*)-2,4-Heptadienal	**P1**, **P3**, **P4**, **P6**	(125) **P1, P3, P4, P6**
(*E*,*Z*)-2,4-Nonadienal		**P1**
(*E*,*E*)-2,4-Nonadienal		**P1, P3**
(*E*,*Z*)-2,4-Decadienal	**P1**	**P1**
(*E*,*E*)-2,4-Decadienal	**P1**	(125) **P1, P6**
Ketones		
Acetone	(1)	
2-Heptanone		**P6**
1-Octen-3-one	**P4**	**P4**
2-Octanone		**P1, P6**
3-Octanone		**P1, P3, P6**
3-Octen-2-one		**P1, P3, P6**
3,5-Octadiene-2-one		**P1, P3, P6**
6-Undecanone		**P1**
β-ionone		**P1**
Alcohols		
1-Propanol	**P6**	
1-Pentanol		**P1, P3, P6**
1-Hexanol		**P1, P3, P6**
1-Octen-3-ol	**P4**	**P1, P3, P4, P6**
Acids		
Acetic acid		(125)
Propanoic acid	**P4**, **P6**	**P4**, **P6**
Hexanoic acid	**P6**	**P6**
Furans		
2,5-Dimethyl furan	**P4**, **P6**	**P1, P3, P4, P6**
2-Pentyl furan	**P1**, **P3**, **P6**	**P1, P3, P6**

There is no distinguishing between (*E*)- and (*Z*)- isomers of the 1-propenyl radicals in **Table 7.1**, as most authors do not indicate which isomer is detected. This is probably because many authors state that only the (*E*)-isomer of the cysteine sulfoxides is present, see section 8.1.1.

The different results of the detection of aroma compounds are probably closely connected to techniques of analyses as described in section 7.3, variety of the leek, growing conditions, and processing if any. The last column in **Table 7.1** clearly shows that the aroma composition changes during frozen storage of leek. Chapter 10 outlines factors which influence aroma composition during storage.

Table 7.1 does not display any ratio between the individual compounds. Abraham et al. (1) reported the total amount of alk(en)yl sulfides distributed as following: 2% di-2-propenyl sulfide, 2% methyl 2-propenyl disulfide, 3% propyl 2-propenyl disulfide, 38% dipropyl disulfide, and 54% methyl propyl disulfide. **Papers 1**, **3**, and **6** found a different distribution in the fresh 15 mm slices, as the three first mentioned compounds together constituted under 1% of the total alk(en)yl sulfides. Dipropyl disulfide corresponded to 38-55% and the major difference was methyl propyl disulfide which constituted 8-14%. Methyl propenyl disulfide represented 10-15% and propyl propenyl disulfide 6-7% (**paper 3** and **6**); these compounds were not detected by (1).

The exact amounts of the different compounds or the ratio between them, however, do not totally reveal the importance of the compounds because of different threshold values and retention by the food matrix. The contribution of the individual compounds to the aroma profile of the product is essential to know in the evaluation of the impact of processing methods.

7.2. Odor active aroma compounds in leek

Volatile sulfur compounds are generally present in trace amounts in foods, but as they normally have low threshold values, they are very important to the aroma. Little information is available about the odor active aroma compounds in fresh leek or other *Allium* species. To my knowledge four papers (125, 143, 144, 172) are concerned with determination of odor active aroma compounds in leek, but none of them are done on fresh leek. **Paper 4** determines the odor active aroma compounds in fresh leek and in frozen unblanched and blanched leek stored for 12 months.

Some authors determine the importance of the compounds on the basis of concentrations, which is misleading because of different threshold values, and this gives rise to disagreement about which compounds are important. Some authors (6, 14, 48) agree that thiosulfinates and thiosulfonates are characteristic for freshly cut onions, as they are present in the headspace of cut onions, but not in the heat treated onions (100). The thiosulfinates generally have a higher threshold value than the sulfides and the thiosulfonates. Methyl (*E*)-1-propenyl thiosulfinate has threshold levels of 0.01 ppm, propyl 1-propenyl thiosulfinate ((*E*) or (*Z*)) has 0.01 ppm, whereas dipropyl disulfide and methyl 1-propenyl disulfide have threshold values of approximately

0.002-0.006 ppm and thiosulfonates have threshold values down to 0.001 ppb (14). This means that the formation of sulfides and thiosulfonates from thiosulfinates, which is described in section 8.1.2, will alter the aroma profile of the leek.

Many of the sulfur compounds are described as *Allium* aroma in the literature. Compounds possessing leek aroma are propanethiol, methyl propyl disulfide, propyl 1-propenyl disulfide, propyl 2-propenyl disulfide, methyl propyl trisulfide, and dipropyl trisulfide (185). Propyl thiosulfinates is described as fresh onion (16), dimethyl disulfide as cabbage-like and dimethyl trisulfide as cooked cabbage-like (97). 3,5-diethyl-1,2,4-trithiolane is described as garlic-like (75). Replacement of oxygen by sulfur in a volatile compound often changes the odor properties from practically no odor to powerful and sometimes unpleasant odor (153), e.g. from water to hydrogensulfide or from methanol to methanethiol. Some of the sulfur compounds reported in **paper 4** were described as rotten (methyl propyl disulfide, methyl 1-propenyl disulfide, and dimethyl trisulfide) and sourish or solvent (propyl 1-propenyl trisulfide).

Many of the C_6 and C_9 aldehydes and alcohols have a green note when present in minor concentrations (72) as they are in fresh leek, but high concentrations of especially hexanal can give rancid flavor (87). Hexanal can have an undesirable odor even when present in lower concentrations though, and this can be the limiting factor of the shelf life of many foods (74). (*Z*)-3-hexenol, hexanal, (*E*)-2-hexenal, and (*Z*)-3-hexenal all possess green notes, whereas (*E*,*E*)-2,4-decadienal and (*E*)- and (*Z*)-2-nonenal are described as fatty (66).

Paper 4 demonstrated by gas chromatography olfactometry (GC-O) that there is no single key impact compound in fresh leek slices, but rather a lot of mainly sulfur compounds, but also aldehydes which contribute to the aroma impression of fresh leek. Petersen et al. (125) found the same for unblanched leek frozen for 3 months. The 10 most important aroma compounds in fresh leek are displayed in **Table 7.2**.

Table 7.2. The ten most important[a] odors detected by GC-O analysis of fresh leek slices. Modified after **paper 4**.

No	Compound	NIF[b]	SNIF[c]
1	Dipropyl disulfide	100.0	9.5
2	Methyl 1-propenyl disulfide*	100.0	8.7
3	Pentanal	100.0	7.7
4	Decanal	100.0	6.0
5	Propyl 1-propenyl disulfide*	100.0	5.7
6	2,5-Dimethyl furan	100.0	4.5
7	Unknown 1	93.3	8.2
8	Unknown 2	93.3	7.9
9	1-Octen-3-ol	93.3	4.4
10	Dimethyl trisulfide	93.3	3.4

[a]The importance was evaluated by the NIF value and if equal the SNIF value. [b]NIF = number of judges in percentage detecting the odor at the peak. [c]SNIF = summed minutes that one peak lasted. *(*E*)- or (*Z*)-isomer.

Both dipropyl disulfide and methyl propenyl disulfide are found in high concentrations in fresh leek by **paper 1**, **3**, and **6**. Dipropyl disulfide is the most abundant sulfur compound in these investigations, and this together with the low threshold value makes this compound important even though some aldehydes are present in higher concentrations.

7.3. Techniques of volatile analysis of *Allium* species

The choice of technique to use when investigating aroma compounds in *Allium* has given rise to many papers (5, 6, 13-15, 21-24, 48, 81, 82). The reason is the formation of thiosulfinates in the freshly cut *Allium*, and the difficulties connected to their detection. Even though it is generally accepted that the spontaneous headspace around freshly cut *Allium* is consisting of mainly thiosulfinates, many investigations do not detect these compounds (**Table 7.1**). This is due to the processing of the product, the sample preparation, the isolation technique and the separation technique. These compounds are critical to analyze both because they are reactive, which means that the time factor is important, and because they are heat labile. This demands very quick and careful treatment both during the isolating of the aroma compounds and the identification procedure.

The isolation procedure has been discussed both in the relation to the sample preparation and the technique of isolation. The many reported isolation techniques have been: solid-phase microextraction (SPME) (5, 81, 82), vacuum distillation (14), extraction (14, 15, 48), supercritical fluid extraction (21-23), cold trapping (48, 81), and trapping on adsorbent (48). Auger et al. (6) claim that thiosulfinates are stable in gas phase, but in liquid phase they rearrange to more stable components as polysulfides and thiosulfonates, which means that the isolation procedure should be done on cut dry samples. Block et al. (14), however, reported relative stability of thiosulfinates in water probably because of an excellent hydrogen-bonding ability of thiosulfinates. They described a simple vacuum distillation procedure for effective isolation of the volatiles.

By using different isolation techniques Ferary & Auger (48) stated that the true odor of leek contains as sulfur compounds only thiopropanal-*S*-oxide, seven thiosulfinates and maybe cepaenes and zwiebelanes and that other sulfur compounds detected are artifacts. They concluded that cold trapping of the volatiles from fresh chopped leeks not mixed with water is the best sampling method, but they also declared that all the sampling methods can produce artifacts, and especially in *Allium* aroma where numerous rearrangements can occur.

The debate on separation techniques has involved mainly HPLC and GC. Block et al. concluded in two papers (14, 15) that the use of GC-MS in detecting thiosulfinates is critical as the heating in the injection port and the detector will cause decomposing. This can partly be overcome by using a short packed wide bore column and temperatures below 100°C, but also longer columns gave reasonable results. HPLC was found to provide the best qualitative measure of the thiosulfinates, which is in agreement with (48). Calvey & Roach (24) reported that a variety of thiosulfinates can be analyzed by GC-MS using wide-bore open-tubular columns and that neither

HPLC nor GC-MS techniques can provide a full characterization of the flavor aroma compounds in freshly cut *Allium*.

In the effort to detect thiosulfinates in this laboratorium several approaches were tested. Dynamic headspace was done on freshly cut leek slices without blending in water and the temperature in the inlet, the oven and the interface to the GC-MS was lowered to 80-90°C, but none of this succeeded in detecting any thiosulfinates (unpublished data). When investigating stored leek slices, however, thiosulfinates formed during processing and storage of the leeks will probably also decompose and will therefore not be present in the tissue at the time of the analysis.

8. Formation of Aroma Compounds

The intact *Allium* cells do not possess any odor as the volatile compounds originate from enzymatic processes that are compartmentalized. Tissue damage such as cleaning and / or cutting the plant will ruin the cell structure and bring together the precursors of the aroma compounds and the requisite enzymes, and consequently numerous of volatile compounds are formed instantly (48, 100, 103, 104, 179).

The formation of volatile compounds in *Alliums* principally consists of two biochemical pathways; those compounds formed from the action of alliinase upon *S*-alk(en)yl cysteine sulfoxides (mainly sulfur-containing compounds) and those compounds generated from unsaturated lipids via the lipoxygenase pathway (primarily carbonyl compounds). During storage formation of aroma compounds via autoxidation of lipids is also a possibility depending on processing and storage conditions. Factors that can affect the content of aroma compounds in leek during processing and storage are described in chapter 10.

8.1. *S*-Alk(en)yl cysteine sulfoxides derived compounds

The distinct pungent aroma of freshly cut leek and other *Allium* species is dominated by numerous sulfur-containing volatile compounds originating from the decomposition of the odorless nonvolatile precursors *S*-alk(en)yl cysteine sulfoxides (ACSO) by the action of alliinase (see section 9.1) (19, 96, 103, 188), as shown in **Figure 8.1** and **Figure 8.2**, section 8.1.2.

8.1.1. Precursors

The different, characteristic odor of the individual *Allium* species arises because of different occurrence of the nonvolatile ACSO (53, 145, 182). At least five to six cysteine sulfoxides occur commonly in *Allium*; these are (+)-*S*-methyl-L-cysteine sulfoxide (MCSO), (+)-*S*-propyl-L-cysteine sulfoxide (PCSO), (*E*)-1-(+)-*S*-propenyl-L-cysteine sulfoxide ((*E*)-1-PeCSO), (*Z*)-1-(+)-*S*-propenyl-L-cysteine sulfoxide ((*Z*)-1-PeCSO) (only reported by (48)), and 2-(+)-*S*-propenyl-L-cysteine sulfoxide (2-PeCSO) (48, 93, 96, 182). (+)-*S*-Ethyl-L-cysteine sulfoxides (ECSO) was believed not to be present in *Allium* species, but the compound has recently been identified in garlic and leek (98). (+)-*S*-butyl-L-cysteine sulfoxides (BCSO) have not been detected in *Allium* (96, 182) and only the (+)-L-isomers of ACSO have been found in nature (171).

MCSO seems to be the most widely distributed ACSO, and it probably occurs in all *Allium* species (182). The abundance of the other cysteine sulfoxides is species specific as onion possess MCSO, PCSO, and (*E*)-1-PeCSO, but not the 2-PeCSO whereas garlic contains the MCSO, ECSO, PCSO, and 2-PeCSO (predominantly), but not the two *S*-1-propenyl isomers (188). In leek MCSO, ECSO, PCSO, (*E*)-1-PeCSO, and 2-PeCSO have been reported (47, 148).

Freeman & Whenhan (53) determined the ratio of sulfoxides in leek as 62.4% of PCSO, 29.3% of MCSO, 8,3% of (*E*)-1-PeCSO, and 2-PeCSO was detected in trace amounts. Approximately

the same distribution was reported by Lancaster (100). Yoo & Pike (195) on the contrary found the presence of MCSO (8.1%) and (E)-1-PeCSO (91.9%) in leaves of leek but no PCSO. This could be due to the distribution of precursors in the plant, which is not uniform. The flavor precursor concentration in young leaf tissue is found to be higher than in old leaf tissue both in onion and leek (100), and inner leaves contain more thiosulfinates than the outer leaves (195). The total amount of precursors in leek was determined to 2.48 mg/g fresh leek (195). The variety of the species also influence on the production of aroma compounds as the amount of precursors is genetically determined (100).

Part of the precursors is present as γ-L-glutamyl peptides which have to be liberated by γ-L-glutamyl transpeptidases before alliinase is able to react on them (146). The two major γ-L-glutamyl peptides in onion bulbs are storage compounds during wintering. The sprouting process increases activity of the hydrolytic transpeptidase, and the cysteine sulfoxides are liberated (17), but still not available for the alliinase reaction as enzyme and substrate are compartmentalized, see section 9.1.1. Breu (19) states that 90% of the soluble organic-bound sulfur is present in the form of γ-L-glutamyl peptides, but according to Whitaker (182) up to 50% of the cysteine sulfoxide is present as γ-L-glutamyl derivatives.

Basis for biosynthesis of these cysteine sulfoxides are amino acids; serine and the two sulfur containing amino acids cysteine and methionine. These last two compounds are present in low concentrations in *Allium*, which is an indication of their rapid metabolism (47). MCSO is thought to be formed both from thioalkylation of serine and from methylation of cysteine (100). Cysteine is also an intermediate in the biosynthesis of (E)-1-PeCSO, and serine together with methionine are precursors of PCSO, and 2-PeCSO (47, 182).

8.1.1.1. Impact of fertilizers on the formation of sulfur compounds

Fertilizing with sulfur-containing salts is important both for the growth of the plants and for the development of aroma compounds. An investigation by Lancaster et al. (102) showed that the pyruvate level was low when the sulfur supply during growth of onion was low, whereas high sulfur supply gave higher pyruvate level. This was supported by Coolong & Randle (34) for onion and by Freeman & Mossadeghi (51) both for onion and garlic. Production of mild and not so pungent onions can be achieved by reducing the supply of sulfur at the right time of the growing season (136).

The biosynthetic pathway begins with the absorption of sulfate, which after a reduction is assimilated into cysteine (94). Available sulfur is used primarily for growth and when these requirements have been met, sulfur is available for incorporation into the biosynthesis of flavor precursors (100). Exogenous sulfate fertilization influences greatly the level of sulfur components in *Alliums* species, but these sulfur compounds are not evenly distributed in the plant as the content increases from the outer tissue to the inner tissue (47).

8.1.2. Volatile products

The products are pyruvate, ammonia, and various sulfenic acids depending upon which of the radical is present on the cysteine-sulfoxide (**Figure 8.1**, Scheme 1). These sulfenic acids are transient intermediates, as they are highly reactive (12, 47, 48, 153, 165, 182) and will quickly combine to form thiosulfinates (**Figure 8.1**, Scheme 2) via a bimolecular condensation reaction. Thiosulfinates can either be symmetrical, if two of the same sulfenic acids condensate or asymmetrical if two dissimilar sulfenic acids join (47). The most important organosulfur compounds in the *Allium* tissue are probably the thiosulfinates and the thiopropanal-*S*-oxide since practically all other organosulfur volatile compounds are originating from these compounds (155).

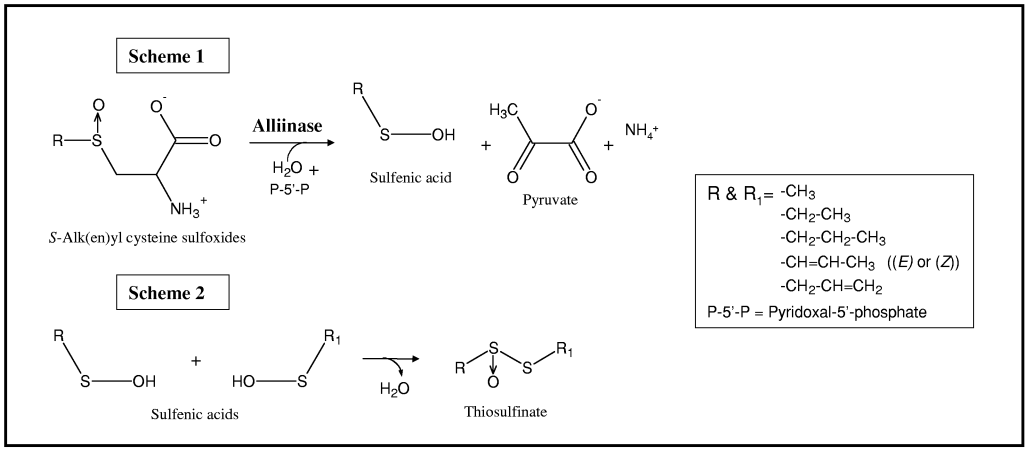

Figure 8.1. Formation of thiosulfinates in leek and other *Allium* species. Modified after **Paper 1**.

Thiosulfinates are pungent-smelling compounds responsible for the odor of freshly cut leeks (14, 48), but as they are relatively unstable (47, 96), they will rearrange to form disulfides and thiosulfonates (**Figure 8.2**, Scheme 1). Thiosulfonates are expelling sulfur dioxide to yield the corresponding monosulfide, and disulfide can rearrange to form mono and trisulfides, so the final products of the reaction will end up being a combination of mono- and polysulfides with all of the possible combinations of the radicals listed in **Figure 8.1**.

As seen in **Table 7.1**, not all of the combinations are commonly found as the ethyl-radical has not been detected in any thiosulfinates, and it is only found in one compound (ethyl 1-methylethyl disulfide) (**paper 1**, **3** and **6**). **Paper 6** on the other hand reported the finding of propyl butyl disulfide and propyl pentyl disulfide; these compounds were identified by MS alone, but with a high certainty. These compounds are not reported by other authors, but may be rearrangement products from other sulfides.

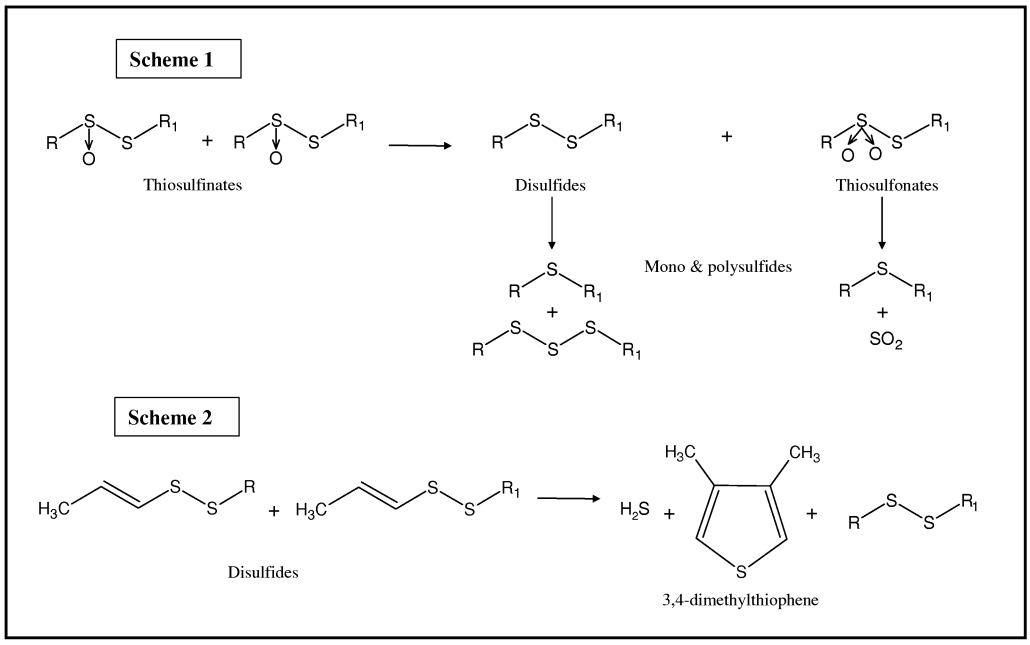

Figure 8.2. Formation of sulfur-containing aroma compounds in leek and other *Allium* species. Modified after **Paper 1** and (16). R & R_1 = methyl-, ethyl-, propyl-, 1-propenyl, or 2-propenyl- in scheme 1 and methyl- or propyl- in scheme 2.

An alternative route is the formation of dimethylthiophenes (**Figure 8.2**, scheme 2) if the radicals on the disulfides are methyl-propenyl- or propyl-propenyl-. This is not very common in freshly chopped onion, however, heating promotes this process (182, 189). The major product formed in onions by this alternative route is 3,4-dimethylthiophene; about 10% is 2,4-dimethylthiophene, and less than 1% is the 2,5-dimethylthiophene isomer (16). Dimethyl disulfide and dipropyl disulfide are also formed from these reactions.

8.1.2.1. Formation of carbonyl compounds from sulfenic acid

(*E*)-1-propenyl sulfenic acid can apart from taking part in formation of polysulfides also rearrange to thiopropanal-*S*-oxide (the lachrymatory factor) (**Figure 8.3**). This compound is present in onion, where it is formed almost exclusively (5), and leek, but not in garlic which lack the (E)-1-propenyl-cysteine sulfoxide. The lachrymatory potency of leek was determined to be moderate strong (53). Thiopropanal-*S*-oxide is also unstable and rearrange spontaneously to form propanal and sulfur (16). As shown in **Figure 8.3** two molecules of propanal can form 2-methyl-2-pentenal by an aldol condensation and subsequent dehydration. 2-methyl-2-butenal can be formed in the same way by condensation of propanal and ethanal (31, 47). Ethanal can arise from the conversion of pyruvate and can further react to form (*E*)-2-butenal (182).

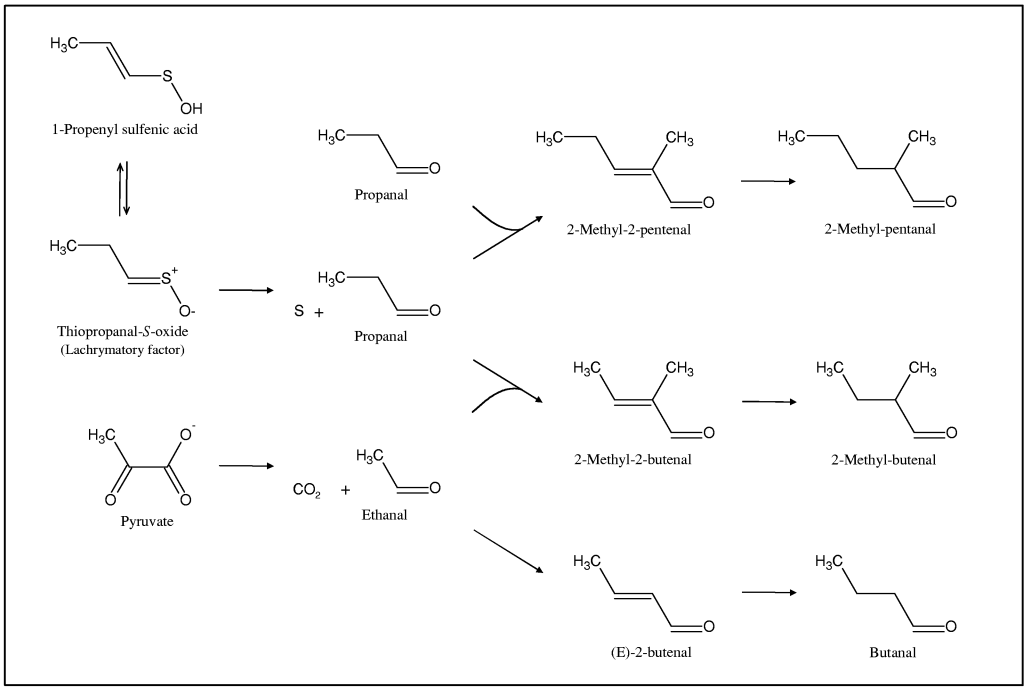

Figure 8.3. Formation of carbonyl compounds from thiopropanal-*S*-oxide. Modified after (16).

3,5-diethyl-1,2,4-trithiolane is postulated to form via decomposition and rearrangement of 2-PeCSO (197) but 2-PeCSO is abundant in traces in leek. Another possibility could be an alternative reaction of disulfides to the one shown in **Figure 8.2**, scheme 1, if the two radicals on the one disulfide are propyl-. The trithiolane is formed by a cyclization of the one disulfide where one sulfur atom from the other disulfide enters the cyclic ring and with that produces a monosulfide. A third possibility mentioned by Block (12) is the trapping of hydrogen sulfide by two molecules propanal to form 3,5-diethyl-1,2,4-trithiolane.

8.2. Lipid derived compounds via the lipoxygenase pathway

The other important pathway in the production of aroma compounds in *Allium* is the lipoxygenase (LOX) pathway. LOX, hydroperoxide lyase (HPL), and alcohol dehydrogenase (ADH) are important enzymes in synthesis of volatile compounds responsible for the green or grassy notes in fruits and vegetables (193), and for off-flavor when present in higher concentrations. LOX catalyzes the peroxidation of polyunsaturated fatty acids originating from cell membranes (57). This leads to the formation of a cascade of mainly C_6 and C_9 aldehydes and alcohols by the action of HPL and ADH (142).

8.2.1. Precursors

Precursors of the lipoxygenase catalyzed reactions are polyunsaturated fatty acids with a (*Z*,*Z*)-1,4-pentadiene moiety. These polyunsaturated fatty acids originate from triglycerides, phospholipids or glycolipids, where they need to be liberated by acyl hydrolases before the oxidative degradation (142). These precursors are not very abundant in *Allium* species, as the total fat content in leek is 0.2-0.3% w/w (7, 47), of which linoleic acid constitutes 32% of the total fatty acids and linolenic acid constitutes 47% of the total fatty acids. Arachidonic acid was not reported although this fatty acid most likely also exists in the leek tissue in minor concentrations. Cassagne et al. (26) demonstrated the biosynthesis of very long chain fatty acids (at least 20 carbon atoms) in leek epidermis, and detected among others arachidonic acid. The abundance of very long acids is low, but they are important in means of wax-forming, and furthermore they might be able to act as precursors of aroma compounds although no literature has been found on that subject.

Free fatty acids do not accumulate in healthy plant tissue, and for that reason the initial phase in the degradation process is the liberation of free fatty acids by lipolytic enzymes (57). As described in details in section 9.2.3 concerning the substrate specificity of LOX, it is mainly linoleic acid and α-linolenic acid that act as substrates in plant tissue. The metabolism of linoleic acid and linolenic acid by the lipoxygenase pathway are shown in **Figure 8.4** and **Figure 8.5** respectively. The first part of the reactions is the incorporation of a peroxide group in the fatty acid to form a hydroperoxide. The actual mechanism of LOX catalytic activity is described in section 9.2.2, but C9-hydroperoxides and C13-hydroperoxides are produced.

8.2.2. Volatile products

The primary volatile product formed from the HPL catalyzed reactions upon the C13-hydroperoxide of linoleic acid (13-HPODE) is hexanal and C9-hydroperoxide of linoleic acid (9-HPODE) yields (*Z*)-3-nonenal (**Figure 8.4**). 13-HPODE also produces the non-volatile oxoacids 12-oxo-9-(*Z*)-dodecenoic acid and 9-HPODE produces 9-oxononanoic acid. The C13-hydroperoxide of linolenic acid (13-HPOTE) forms (*Z*)-3-hexenal and C9-hydroperoxide of linolenic acid (9-HPOTE) derives (*Z*,*Z*)-3,6-nonadienal (**Figure 8.5**). The hydroperoxides of linolenic acid produce the same oxoacids as mentioned above.

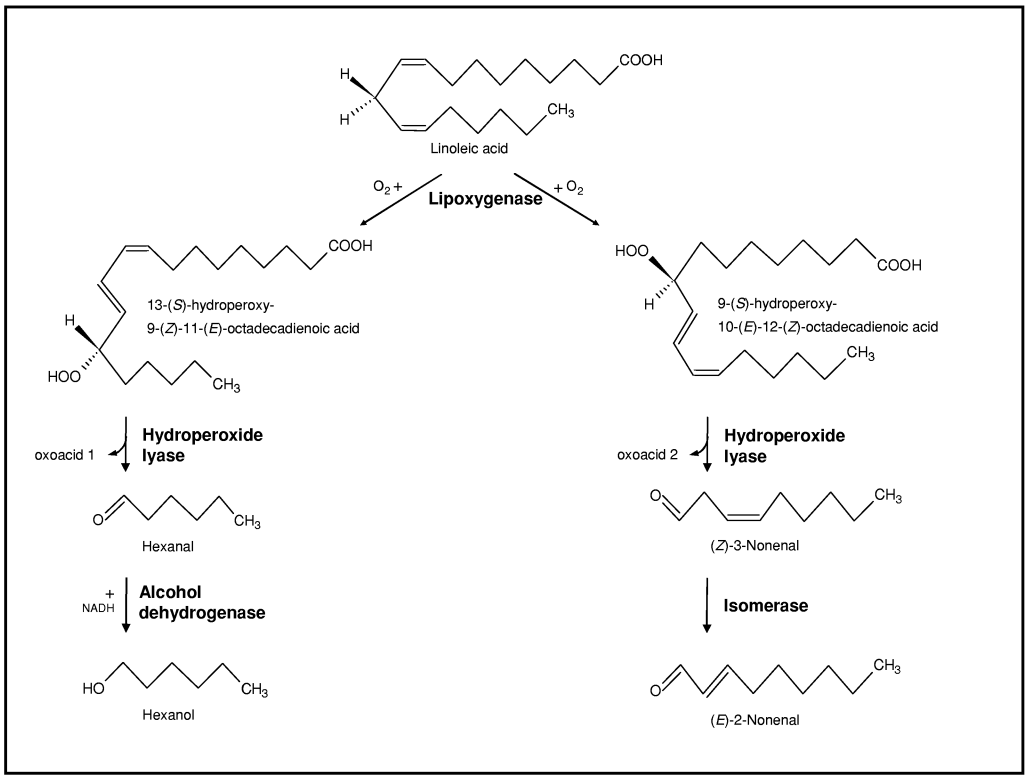

Figure 8.4. Formation of volatile compounds from linoleic acid via the lipoxygenase pathway. Oxoacid 1 = 12-oxo-9-(*Z*)-dodecenoic acid and oxoacid 2 = 9-oxononanoic acid.

All these volatile compounds can be reduced further to alcohols by ADH. Primary HPL products containing a double bond such as (*Z*)-3-hexenal can also be converted by allylic isomerization to the trans isomer (*E*)-2-hexenal (191). Isomerization of (*Z*)-3-hexenal to (*E*)-2-hexenal occurs either spontaneously or enzymatically (166). Most plant extracts catalyze the (*Z*)-3- to (*E*)-2-isomerization of monounsaturated aldehydes although with variable capacities. Evidence suggests that this isomerization is catalyzed by a separate isomerase enzyme (128), but HPL catalyzing the reaction is also possible (175). Investigations on HPL from tea leaves have shown though that HPL retains the (*Z*)-configuration in the substrates and the isomerization to the (*E*)-isomer occurs after the cleavage reaction (70).

Haslbeck & Grosch (69) found that soybean lipoxygenase is also capable of producing the 8-, 10-, 12- and 14-hydroperoxides of linoleic acid but only in minor amounts. This could lead to the formation of (*E*,*Z*)-2,4-decadienal, (*Z*)-3-nonenal, hexanal and pentanal. (*E*,*Z*)-2,4-decadienal and (*E*)-2-octenal are most likely also formed by lipoxygenase initiated reactions in raw shredded potatoes (84).

The model experiment reported in **paper 5** demonstrated that crude enzyme extract from leek mixed with linoleic acid formed significantly most hexanal, heptanal, (*E*)-2-heptenal, (*E*)-2-

octenal, (*E*,*E*)-2,4-decadienal, pentanol, and hexanol when compared to linolenic acid and blank samples. Reaction upon linolenic acid resulted in significantly most (*E*)-2-pentenal, (*E*)-2-hexenal, (*E*,*Z*)-2,4-heptadienal, (*E*,*E*)-2,4-heptadienal, and butanol.

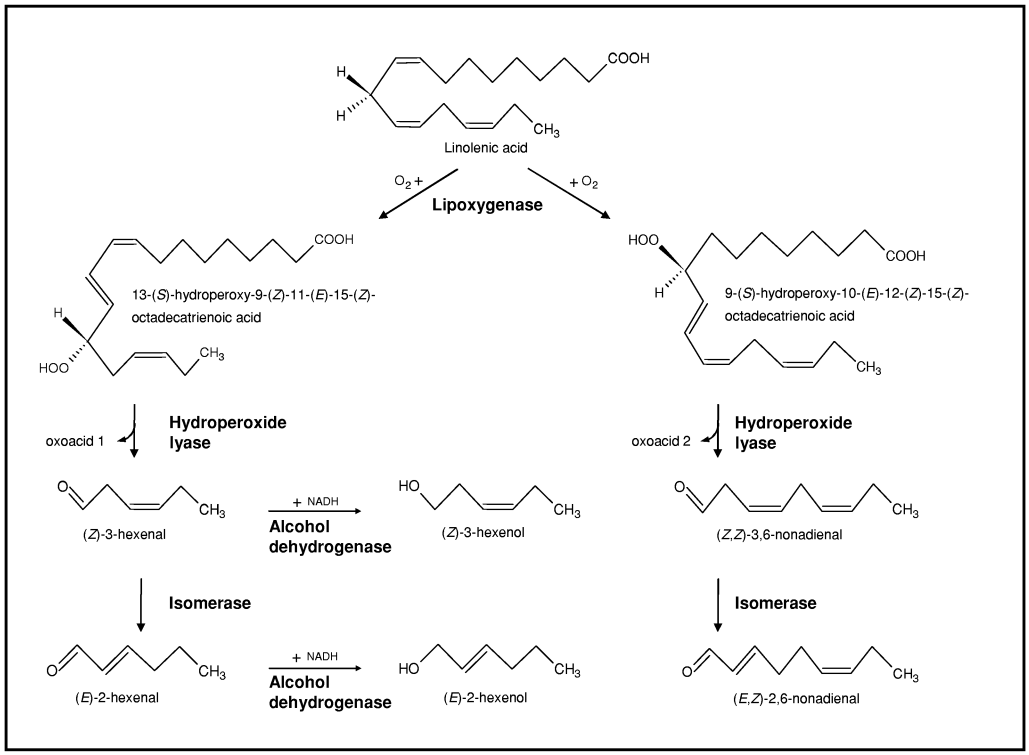

Figure 8.5. Formation of volatile compounds from linolenic acid via the lipoxygenase pathway. Oxoacid 1 = 12-oxo-9-(*Z*)-dodecenoic acid and oxoacid 2 = 9-oxononanoic acid.

HPL in mushroom initiates the production of 1-octen-3-ol by action on 10-(*S*)-hydroperoxy-8-(*E*)-12-(*Z*)-octadecadienoic acid (190) and 1-penten-3-one, 1-penten-3-ol, and (*Z*)-2-pentenal are all further products of LOX cleavage of C-13-hydroperoxides (173). Aldehyde dehydrogenase can oxidize the aldehydes to acids (183), which might be the reason for the detected acids in leek.

8.3. Lipid derived compounds via autoxidation

The compounds derived from enzymatical metabolism of fatty acids can also be generated by autoxidation during the storage period (65). Enzymatical production of volatiles is more selective than autoxidation and thus usually results in a narrower range of volatiles. Only unsaturated fatty acids are autoxidized at normal storage conditions (65), and the rate of autoxidation increases with the degree of unsaturation; linoleic acid is oxidized 10 times faster than oleic acid and linolenic acid 23-30 times faster (76). While the enzymatically produced hydroperoxides in most cases yield one hydroperoxide as the predominant product, non-enzymatical oxidation of unsaturated lipids yield a mixture of hydroperoxides which differ in the position of the OOH group and also in the geometrical isomerism of the double bonds (30, 59). As the number of double bonds increases, the number of oxidation and oxygen addition sites increases proportionately (63).

The initiation reaction is the removal of hydrogen attached to an allylic carbon atom to form a radical in the presence of an initiator, which can be light, heat or the presence of metal ions. Under normal oxygen pressure the radical reacts rapidly with oxygen to form the peroxy radical, which will react with more unsaturated lipids to form hydroperoxides (76). These hydroperoxides are unstable and decompose to an alkoxy radical and a hydroxyl radical by cleavage of the –OOH group. Alkoxy radicals are further transformed to yield aldehydes. Aldehydes can be produced by cleavage of the lipid molecules on either side of the radical in contrast to HPL catalyzed reactions that are specific towards one side (section 9.3.2).

Autoxidation of linoleic acid produces C9-hydroperoxide and C13-hydroperoxide, whereas linolenic acid in addition also produces C12-hydroperoxide and C16-hydroperoxide (76). An alternative route for the hydroperoxides is the hydroperoxide lyase (HPL) catalyzed breakdown to aldehydes and oxoacids (section 9.3), but this is probably only possible for the C9-hydroperoxides and the C13-hydroperoxides due to the specificity of HPL.

Hexanal and 2,4-decadienal are the primary oxidation products of linoleic acid, from the C13-hydroperoxide and the C9-hydroperoxide, respectively. Further degradation of 2,4-decadienal via autoxidation leads to formation of 2-octenal, which is converted to hexanal (84). The autoxidation of linolenic acid yields (*E*,*Z*)-2,4-heptadienal as the major product (65). 2-pentylfuran is derived from the autoxidation of linoleic acid, if the conjugated diene radical formed from the cleavage of the C9-hydroperoxide reacts with oxygen. This produces a vinyl hydroperoxide that can undergo cyclization via the alkoxy radical to yield 2-pentylfuran (76).

Table 8.1 summarizes the volatile compounds produced by autoxidation at moderate temperature according to (65). Arachidonic acid is a ω6 fatty acid as linoleic acid and therefore many of the produced carbonyl compounds with less than 11 carbon atoms will be the same (10, 65). The major compounds produced are also hexanal and 2,4-decadienal in almost equal

amounts, whereas for linoleic acid the ratio between hexanal and 2,4-decadienal is about 12:1 (65).

Table 8.1. Volatile compounds derived from the autoxidation of linoleic acid, linolenic acid, and arachidonic acid at moderate temperature. Modified after (65).

Compound	Linoleic acid	Linolenic acid	Arachidonic acid
Propanal		X	
Pentanal	X		X
Hexanal	X	X	X
Heptanal	X		
Octanal	X		
(*E*)-2-Butenal		X	
(*E*)-2-Pentenal		X	
(*Z*)-2-Pentenal		X	
(*E*)-2-Hexenal		X	
(*E*)-3-Hexenal		X	
(*Z*)-3-Hexenal		X	
(*E*)-2-Heptenal	X	X	X
(*E*)-2-Octenal	X		X
(*Z*)-2-Octenal	X		X
(*E*)-2-Nonenal	X		
(*E*)-3-Nonenal	X		
(*Z*)-3-Nonenal	X		X
(*Z*)-2-Decenal	X		
(*E*,*Z*)-2,4-Heptadienal		X	
(*E*,*E*)-2,4-Heptadienal		X	
(*E*,*E*)-2,4-Nonadienal	X		
(*E*,*Z*)-2,6-Nonadienal		X	
(*E*,*Z*)-2,4-Decadienal	X		X
(*E*,*E*)-2,4-Decadienal	X		X
1-Octen-3-one			X
3-Octen-2-one			X
3,5-Octadien-2-one		X	X
2-Pentyl furan	X		

Investigations on blanched and unblanched leek slices stored frozen for one year in the dark indicated that mainly enzyme activity was responsible for the production of aldehydes, because unblanched samples produced almost four times higher concentrations of aldehydes than blanched samples did (**paper 6**).

9. Enzymes Related to Aroma Formation

Alliinase is the most important enzyme responsible for the development of flavor compounds in fresh *Allium* species (179), but as described in chapter 8 the enzymes from the lipoxygenase pathway are also contributing to the development of flavor. The abundance of the enzymes, their catalytic activity and substrate specificity, and their different optimum regarding pH and temperature are important to know in the effort to maintain quality of frozen leek slices during storage.

9.1. Alliinase

The enzyme alliinase (EC 4.4.1.4) has been isolated from higher plants, where the genus *Allium* is an important source, but also cabbage, cauliflower and broccoli in the genus *Brassica* possess the enzyme (119); apart from that the enzyme is not very widespread. The metabolic role of alliinase is not fully determined, but the enzyme might be involved in a plant defense mechanism (135). The substrates, *S*-alk(en)yl L-cysteine sulfoxides, are metabolically inert (47), but di-2-propenyl thiosulfinate (allicin), which can be a product of alliinase catalyzed reactions, shows antimicrobial and antifungal activity in garlic cloves, and thereby prevent invasion of the plant (4). Furthermore, the lachrymatory factor thiopropanal-*S*-oxide and other of the produced sulfur compounds are believed to prevent attack from herbivores (187) because of the pungency of the compounds.

Alliinase has been isolated from many different *Allium* species and although the plants belong to the same genus, the alliinases are different in their physical and chemical properties (120, 188). This means that the properties of alliinase origin from one source are not necessary valid for alliinases from other sources. Most of the literature found on alliinases is done on the basis of onion and garlic, whereas leek is not so commonly investigated, which implies that some information is only available for other species than leek. Alliinase from leek is probably more equal to onion alliinase than to garlic alliinase (188).

9.1.1. Localization in the cell

Alliinase is situated in the vacuoles (101). The substrate is endogenous *S*-alk(en)yl-L-cysteine sulfoxides (ACSO), which are present as nonprotein amino acids in the cytoplasm of the protoplasts (101, 188). The vacuoles contain only minor amounts of ACSO (101), and consequently damage of the cell is needed before catalytic activity is possible. Alliinase is evenly distributed in the leek plant, but not more than 0.04% of the total amount of soluble protein by weight in leek is alliinase (108). This is in contrast to onion bulbs where alliinase is representing up to 6% of the soluble protein and garlic cloves, which holds up to 12% alliinase (119).

9.1.2. Catalytic activity

Alliinase (other names: alliin lyase or cysteine sulfoxides lyase) is registered in the enzyme group 4 – the lyases. Alliinase catalyses a β-elimination-deamination reaction in which a double

bond is formed and a C-S bond is cleaved (**Figure 9.1**). Pyridoxal-5'-phosphate (P-5'-P) is needed as a cofactor.

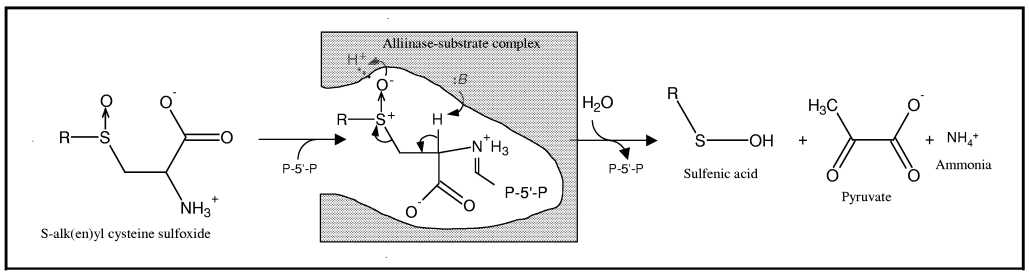

Figure 9.1. Catalytic activity of alliinase. P-5'-P = pyridoxal-5'-phosphate. *:B* = An alkaline group. R = methyl-, ethyl-, propyl-, 1-propenyl- or 2-propenyl. Modified after (11, 19, 109).

The molar ratio of P-5'-P to enzyme is not investigated in leek, but both for onion, garlic, and Chinese chive it is determined to approximately one mole P-5'-P per subunit of the enzyme (109, 118, 169). The first step in the reaction is the cleavage of the C-S bond of ACSO to yield sulfenic acid and α-aminoacryl acid, which is spontaneously hydrolyzed to pyruvate and ammonia (109). P-5'-P activates the binding of the substrate to the enzyme, which is based on electrostatic interactions between the substrates and a metal localized in the active centre of alliinase (19). Alliinase from garlic has 10 cysteine residues, which are all in S-S bridges, and which are essential for activity, as the reduction of them leads to inactivation of the enzyme (4).

The P-5'-P binding site of onion alliinase was identified as lysine 285 in the amino acid sequence (91) and as lysine 280 in Chinese chive alliinase (109) and P-5'-P is probably bound as a Schiff base with the amino group of the enzyme (31). A tyrosine-rich region positioned 60-90 amino acids away from the P-5'-P binding site is probably responsible for the interaction with P-5'-P and the substrate (109). Lohmüller et al. (108) found indications of a hydrophobic region at the active site of the leek enzyme which was essential for the binding of the substrate. After subtraction of hydrogen by an alkaline group, the substrates form pyruvate, ammonia, and sulfenic acids (19). Sulfenic acids are very reactive (188) and quickly rearrange via non-enzymatic reactions to other products (section 8.1), which means that the reaction probably is nonreversible.

The reaction mechanism does not motivate the occurrence of a lag phase, and this has not been described in the literature. Nor did the investigation of alliinase reported in **paper 6** show evidence of a lag phase.

9.1.3. Substrate specificity

The distinct odors of different *Allium* species are believed to be due to different contents of ACSO and to different substrate specificity of alliinases (109). Alliinase might be present in two or more isoforms of differing substrates specificity (104, 119). Leek alliinase show most activity

towards 2-PeCSO (108, 188), but did also show activity against MCSO, PCSO, and ECSO. Only L-cysteine derivatives where the sulfur is oxidized to S→O work as substrates (149, 150), which indicates that the oxygen atom on the sulfur group is important for the cleaving process of the C-S bond. The presence of oxygen at the sulfur atom of the sulfoxides introduces another asymmetrical center in the molecule (151), which might be important. The natural occurring (+)-forms of the L-cysteines are hydrolyzed more rapidly than the other diastereomeric form, and unsaturated cysteine sulfoxides are better substrates than the corresponding saturated derivatives (108, 147). According to Stoll & Seebeck (164) the sulfur atom of cysteine can only be linked to an aliphatic group to act as a substrate, whereas aromatic residues are not metabolized. Lohmüller et al. (108) however, did find some activity against *S*-benzyl-L-cysteine sulfoxide.

The K_m of leek alliinase was 13 mM for ECSO and 55 mM for MCSO (188). This is higher than for onion (5.7 mM for ECSO and 16.6 mM for MCSO) (151), which indicates that onion alliinase has higher affinity for the substrates concerned than leek alliinase has. The specific activity of alliinase was greater at low sulfur supply than at high sulfur supply in onion (102).

9.1.4. Impact of pH and temperature

Conflicting information on the pH optimum of purified leek alliinase has been found. Lohmüller et al. (108) reported a broad pH optimum between pH 6.1 and 6.9, while Won & Mazelis (188) found a pH optimum at 8.0, which is the same as found for onion (169). pH optimum of garlic alliinase was determined between pH 5-8 (163).

Temperature optimum for alliinase in leek was determined to 41°C by (108), and this was accompanied by a great stability against heat. Garlic alliinase showed a temperature optimum of 37°C under standard conditions (163), and onion alliinase demonstrated optimum activity at 40°C (68). The purified alliinase from leek showed great stability against elevated temperatures, as activity was still detectable after 35 min at 60°C or 5 min at 70°C (108). This should mean that alliinase in intact cells would be even more stable against heating. **Paper 6** demonstrated that alliinase has greater heat stability than LOX when heat treated in the intact tissue.

9.1.5. Natural inhibitors

Inhibitors of alliinase can be divided into two classes: those involving pyridoxal phosphate antagonists and those using substrate analogues as competitive inhibitors (100). *S*-alkyl-cysteines are partially competitive inhibitors of onion alliinase, and it is suggested that this inhibition is part of a metabolic control (151). Methionine sulfoxides are competitive inhibitors of alliinase (182). Hydroxylamine is an irreversible inhibitor of alliinase (114), because hydrozylamine is a chemical inhibitor of P-5'-P reactions (109).

9.1.6. Protein characteristics

The purified leek alliinase is a glycoprotein with the major sugars being mannose, glucose and galactose (188). The enzyme is composed of several equal subunits with a molecular weight of 48 000. Two active alliinases have been found in leek with molecular weights of 386 000 and

586 000, respectively, which indicates that the active enzymes are composed of 8 or 12 subunits (188). This is partly in agreement with (108), who found a subunit molecular weight of 50 000 and two active protein fractions, but the molecular weights were 295 000 and 117 000 respectively, indicating up to 6 subunits.

The molecular weight is higher than reported from other species of *Allium*, as the onion holoenzyme has a molecular weight of 200 000 (119) and the garlic holoenzyme has a molecular weight of 180 000 (134). Both enzymes are composed of four equal subunits. Nock & Mazelis (118) found that garlic alliinase consist of two equal subunits with a total weight of 85 000. Clark et al. (33) reported the finding of two subunits with different molecular mass in onion. Two isoforms of alliinase has been reported in onion roots (103).

9.1.7. Assays

The most common assays are based on spectrophotometrical determination of pyruvate either direct or coupled by measuring a product of the further conversion of pyruvate. The most commonly used substrate is ethyl cysteine sulfoxide (ECSO). ECSO was discovered in *Allium* only recently but ethyl-cysteine is commercially available and most alliinases show high activity against the corresponding sulfoxide.

There are two common ways of determining the production of pyruvate. One is the discontinuous assay based on a spectrophotometrical endpoint measurement at 450 nm of the pyruvate produced (188). The sample preparation is done by the total keto-acid method described by (55). The other one is a continuous coupled assay based on lactate dehydrogenase which reacts on pyruvate under the consumption of NADH; the decrease of NADH is followed spectrophotometrically at 340 nm ((103), **paper 6**).

Other methods are: The 2-nitro-5-thiobenzoate method based on the reaction of 2-nitro-5-thiobenzoate with 2-propenyl-thiosulfinate which can be detected spectrophotometrically (113). The 4-mercaptopyridine method where 4-mercaptopyridine reacts with the activated disulfide bond of thiosulfinates, which can be detected spectrophotometrically (114). The 2,4-dinitrophenyl hydrazine method where pyruvate is determined by the derivatization of 2,4-dinitrophenyl hydrazine (167). The thiosulfinate method where thiosulfinate was determined spectrophotometrically by monitoring the absorbance at 515 nm after reaction with N-ethylmaleimide (167).

9.2. Lipoxygenase

The abundance of lipoxygenase (LOX) is widespread in aerobic organisms, and the enzyme has been isolated both from plants, animals, and micro organisms (174). In plants the LOX (EC 1.13.11.12) catalyzed reactions are involved in defense (e.g. pest resistance), senescence, seed germination, stress response, and communication (18, 74, 138, 174), and LOX is said to be a stress-related enzyme. This probably means that the abundance of LOX activity is not related to any particular stage of growth or development of the vegetables. However, LOX activity is found to increase greatly during wounding of the plant tissue (74). Well over one hundred products derived from LOX-generated hydroperoxides have been detected (61). Some of these reactions also lead to the formation of short chain aldehydes and alcohols, which will contribute to the aroma of freshly cut leeks as described in chapter 8. Production of C_6-aldehydes via the LOX pathway is thought to be a disposal route for the removal of toxic hydroperoxides (173).

The lipoxygenase pathway has been the subject of many review papers over the years (42, 60, 61, 64, 184). As legume seeds contain particularly high levels of LOX activity (38) many investigations on LOX in soybeans have been reported, but also potato, tomato, and peas have given rise to many publications. Limited information is available on the activity of LOX in *Allium* species, and only four papers apart from **papers 1-3** and **5-7** have been found. One is concerned with leek (8), two with onion and garlic (73, 129) and one with chives (41). For that reason most of the literature cited is concerned with LOX activity in other plant material than *Allium*.

9.2.1. Localization in the cell

LOX was for a long time thought to be randomly distributed throughout the cytosol, because it is readily solubilized by tissue homogenization, but this was later invalidated, and now it seems possible that different LOX isoenzymes may have specific locations (50). LOX localization in soybean seed showed a dependence on developmental stages, and only in the non-germination stage LOX was present in the cytosol (60). In fresh tea leaves the majority of the lipoxygenase activity is associated with the lamella membrane of chloroplasts (73), and localization in the chloroplasts has also been found for a number of other plants (60). LOX isoenzymes of cucumber are localized in lipid bodies (64), and LOX from tomato was found both in soluble and microsomal membranous compartments (137).

9.2.2. Catalytic activity

LOX is an oxidoreductase (official name is linoleate:oxygen oxidoreductase) which catalyses a two-substrate reaction. LOX catalyses the peroxidative modification of certain polyunsaturated fatty acids having a (*Z*,*Z*)-1,4-pentadiene system by the incorporation of molecular O_2 to form a conjugated (*Z*,*E*) fatty acid hydroperoxide (74, 177). All known plant LOX attack the prochiral center C-11of linoleic acid or linolenic acid (64).

Lipoxygenase contains 1 mol of non-heme iron per mol of enzyme, which is essential for catalytic activity (174). The iron atom is tightly bound to LOX by six ligands of which three are histidines (62). To catalyze a reaction LOX must be in the oxidized form (Fe^{3+}), which is usually done by its own lipid hydro peroxide product (**Figure 9.2**) (64). After oxidation of the iron, a fatty acid is attached to the enzyme followed by the second substrate oxygen (74, 174, 184). The two-substrate reaction apparently involves a ternary complex formed in an ordered manner because oxygen is only able to bind to the enzyme after the fatty acid has already bound. Ca^{2+} ions have been proposed as a factor for optimum lipoxygenase activity as well, but the results are very much related to the origin of the enzyme and are also varying depending on the properties of the isoenzymes (130, 174).

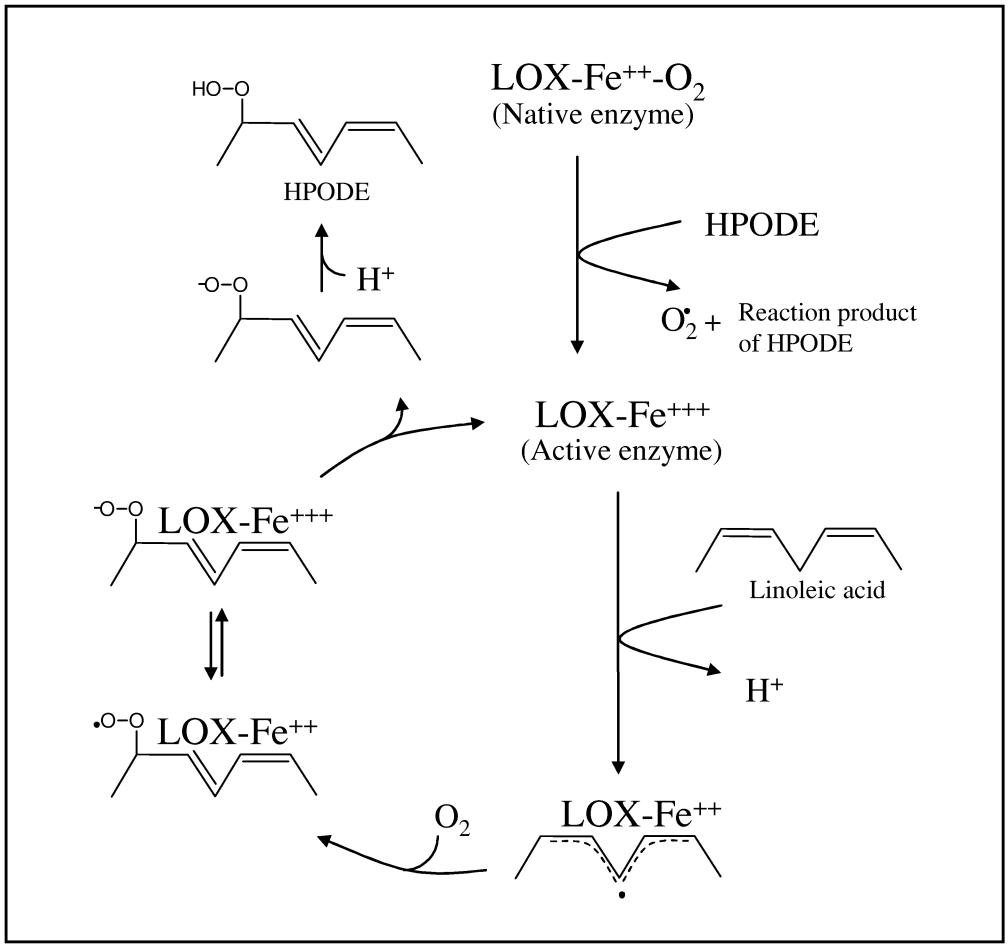

Figure 9.2. Reaction scheme of the activation of soybean LOX and of the catalytic activity. HPODE = hydroperoxide of linoleic acid. Modified after (37, 49, 60).

The catalysis include three stages, first a stereospecific hydrogen removal from the methylene group between the two double bonds, secondly an allylic rearrangement of the resulting free

radical which causes conjugation and cis to trans isomerization of the double bond, and last the binding of molecular oxygen to the formed (*E*,*Z*)-2,4-pentadiene structure (64, 183). The hydrogen abstraction is the rate-limiting step of the overall reaction (74).

Only about 1% of the enzyme is present in the active oxidized form (158). This means that some sort of lag phase could be expected before hydroperoxides are formed and are able to activate more enzymes. **Paper 1** demonstrated a lag phase of 150-200 seconds until maximum activity was reached. The lag phase in vivo might be more pronounced than the one determined in experiments, because the enzyme is already activated during the preparation of the enzyme extract. Veldink et al. (174) stated that only hydroperoxides formed by the enzyme are effective in activating LOX, and the lag phase of soybean LOX was only shortened by the 13-L-isomer of hydroperoxide.

Dioxygen is normally introduced either at the 9- or 13-carbon of linoleic or linolenic acid, which are the predominant substrates in plants (64). The attacked (*Z*)-double bond delocalize non-enzymatic into conjugation with the other (*Z*)-double bond in the 1,4 pentadiene structure and assumes an (*E*)-configuration leading to the formation of the corresponding (*Z*,*E*)-conjugated hydroperoxy-diene or –triene fatty acid (72, 142). The origin of the LOX determines the ratio of 9- to 13-hydroperoxides (61, 138), and the different isoenzymes also give different ratios (59) but in either case the hydroperoxide group has an *S*-enantioconfiguration (142). The hydroperoxide products can be metabolized further by at least four major metabolic routes (50), where only the HPL route is described in this thesis because this leads to the formation of volatiles.

LOX is also able to function in anaerobic conditions, but only if fatty acid hydroperoxides are already present. In this process the hydroperoxide replaces oxygen as the oxidant of iron (60). In the case of linoleic acid this can lead to the formation of pentane and pentanol and other products (49).

9.2.3. Substrate specificity

The required substrate is a methylene-interrupted (*Z*,*Z*)-1,4-pentadiene moiety (138, 139, 184) which is present in the polyunsaturated fatty acids linoleic acid, linolenic acid and arachidonic acid. The geometrical isomers of linoleic acid (9*E*,12*E*), (9*E*,12*Z*) and (9*Z*,12*E*) do not act as substrates (71) probably because they do not fit into the active site of LOX (**Figure 9.3**). Other compounds such as carotene, which holds the pentadiene structure, are known to be oxidized by some plant LOX as well. Investigations with tea chloroplast LOX showed that unsaturated fatty acids with C_8 –C_{12} chains did not act as a substrate, and that only (*Z*,*Z*)-1,4-pentadiene structure at the ω6 and ω9 position gave rise to LOX activity (71). This means that the third double bond in α-linolenic acid cannot take part in the pentadiene structure.

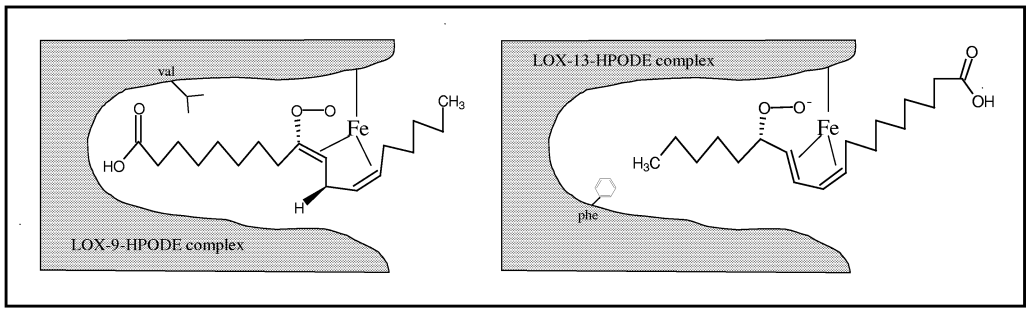

Figure 9.3. Proposed oxidation mechanism of LOX. Figure at the left shows the oxidation at the C-9 position after an initial attack of O_2 from behind the linoleic acid chain and electron transfer via the iron-active site. Figure at the right shows the oxidation at the C-13 position with the completed oxidation product. Val = valine, phe = phenylalanine, HPODE = hydroperoxide of linoleic acid. Modified after (49, 60).

The initial event in the LOX pathway is the release of free fatty acids from glycerolipids such as phospholipids, which apparently are the usual substrates. This is done by lipolytic acyl hydrolases (71). LOX possess a hydrophobic pocket to bind the hydrophobic methylene chain of the substrate and a hydrophilic area, whose substrate requirements are fairly broad (72).

Many LOXs have little reactivity towards fatty acids esterified in glycerolipids. The oxidation at the C-9 position is only possible when the fatty acid is liberated from the glycerol moiety, because, as shown in **Figure 9.3**, the acid part of the fatty acid is inside the active site of the enzyme. Oxidation in the C-13 position is possible with fatty acids in glycerolipids (58). In the bottom area of the substrate-binding pocket a space-filling histidine or phenylalanine residue is situated in the C-13 specific LOX and a valine residue in the C-9 specific LOX (50).

Linoleic acid and linolenic acid are the most abundant polyunsaturated fatty acids in plants and therefore the most likely substrate for LOX activity (138, 178). The experiment reported in **paper 5** showed almost 4 times higher activity against linoleic acid when compared with linolenic acid, and the results also indicated that essentially only the 13-hydroperoxide was produced. LOX from a homogenate from fresh leaves of onion produced approximately the same amount of (*Z*)-3-hexenal and hexanal, which indicates that both linoleic acid and linolenic acid are metabolized (73). This investigation did not look for C_9 compounds, so it is not possible to draw any conclusions regarding the specificity of the produced hydroperoxides.

Investigations on other genus in most cases show high specificity towards producing one hydroperoxide. According to Galliard & Phillips (58) the potato LOX almost exclusively converts linoleic acid into 9-D-hydroperoxyoctadeca-(*E*)-10, (*Z*)-12-dienoic acid (95%) whereas the 13-hydroperoxy isomer was only a minor product (5%). The same is reported for tomato (61), while soybean primarily forms the 13-hydroperoxide.

9.2.4. Impact of pH and temperature

Paper 5 reports a pH optimum in leek of 4.5-5.5 with linoleic acid as substrate, which, to my knowledge, is the only paper concerning pH optimum of LOX in leek. No papers have been found reporting pH optimum in onion or garlic but in chives pH optimum has been determined to 6.0 (41). Variable pH optimum has been found for other plant LOX. Potato LOX has pH optimum in the range of 5.5-6.0 (20, 58, 90, 174), and in soybean the pH optimum is dependent on the isoenzymes. Two isoenzymes have optimum at 6.0-6.2 and the third isoenzyme has optimum at 9.0 (49). LOX in germinating watermelon seedlings showed two pH optima of 4.4 and 5.5 (177) probably also because of different isoenzymes. According to Gardner (61) LOX with pH optima near neutrality often are specific for 9-oxidations, but this was not confirmed for leek LOX by **paper 5**.

LOX is considered as a heat labile enzyme, and **Paper 6** demonstrated that LOX was more heat labile than alliinase, HPL and ADH. Experiments with thermal denaturation of LOX from soybeans showed that the enzymes are better protected against thermal denaturation if substrate binding is possible, which means that liberating of the substrate by cutting into slices would protect the enzymes better than in the intact plant (3). **Paper 2** demonstrated that catalytic activity of LOX was lost in 15 mm leek slices after 3 minutes of water blanching.

9.2.5. Natural inhibitors

Di-2-propenyl disulfide and dimethyl disulfide from garlic extract inhibit soybean LOX irreversible, probably because disulfides can modify the thiol group of cysteine residue present in the active site of LOX (89). The determination of LOX in fresh leek slices as reported by **paper 1-3**, **5**, and **6** clearly indicates that this in not the case with LOX originating from leek, as the activity in the crude extract is high.

A self-catalyzed destruction mechanism is apparently a general phenomenon to all LOX, and it is explained as a result of uncontrolled free radicals generated as side products of the main event of fatty acid hydroperoxidation (60) or by accumulation of excess amounts of enzyme-generated hydroperoxides (74, 79).

Fatty acids containing a (*E*)-double bond are not oxygenated by LOX from soybean but act as competitive inhibitors. Both (*Z*)- and (*E*)-fatty acids are bound to the enzyme, but the initial rate determining hydrogen abstraction is steriocally obstructed in the (*E*)-configuration (79). The inhibition of LOX by α-tocopherol is reported (29). A bonding through the hydrophobic chain of α-tocopherol forms a complex with LOX, which causes the inhibition.

9.2.6. Protein characteristics

Multiple isoenzymes of LOX have been described for many plant species (49), and LOX is present as at least 5 isoenzymes. The molecular weight of plant LOX's is about 100 000 ± 20 000 (60), but no specific information on LOX from *Allium* has been found.

9.2.7. Assays

There are two commonly applied assays to determine LOX activity. One is the spectrophotometrical measurement of the increase in absorbance at 234 nm corresponding to the conjugated diene system in the hydroperoxide product. The other is the measurement of the oxygen consumption with a recording oxygen electrode, usually the Clark electrode princip (42, 192). Both methods can be followed continuously, but proteins and other compounds present in crude and partially purified plant extracts often contribute to strong absorption at 234 nm, which limits the amount of enzyme that can be added to the spectrophotometrical assay (175). This probably makes the oxygen measurement by means of an electrode the most suitable method.

Other assays are based on colorimetric peroxide determination or co-oxidation of suitable substrates (carotene) (174).

9.3. Hydroperoxide lyase

HPL is widespread in the plant kingdom but has so far not been detected in any mammal (64). The enzyme is found in green and non-green tissues and has been isolated from many different parts of the plant such as leaves, roots, seeds, and fruits (45). Relatively small amounts of HPL is present in plant tissue (157). The role of HPL is partly plant defense and partly production of flavors, as HPL is involved in the biosynthesis of volatile aldehydes and alcohols, which are important constituents of the characteristic flavors of fruits, vegetables and green leaves (157).

HPL catalyses the metabolism of hydroperoxides, that are toxic to the plant. HPL is activated by plant injury and may function in a wound response. One product of HPL's cleavage of 13-HPODE and 13-HPOTE – the nonvolatile 12-oxo-9-(*Z*)-dodecenoic acid is a precursor of traumatin, a compound involved in wound healing (166). Many of the produced volatile compounds demonstrate toxicity towards some pathogens and insects, e.g. hexanal and (*E*)-2-hexenal, which indicates that these reactions are part of plant protection (35, 176). Attacking insects are wounding the tissue by sucking the plant and by that starting the reaction of toxin production (173). In absence of plant injury the normal pathway of fatty acid hydroperoxide metabolism is probably by hydroperoxide dehydrase (HPD) (178). This leads to the biosynthesis of jasmonic acid which possesses growth regulating properties.

9.3.1. Localization in the cell

No information about the localization in *Allium* tissue is, to my knowledge, available, but most of the HPL's are membrane bound enzymes (64). In green tissues the major site of localization is the chloroplast thylakoid membranes, but in non-green tissue other sites are described (45, 178). HPL from cucumber flesh has been located in three separate membranes; plasma, golgi and endoplasmic reticulum (181). HPL in fresh green tea leaves is bound to the lamella membrane of chloroplasts, where they form part of an enzyme system together with LOX (73). Riley et al. (137) reported that virtually all HPL present in tomatoes are associated with microsomal membranes which also contain LOX. It is suggested that the HPL activity in green bell pepper is partly regulated by the organization of the membrane around the enzyme (157).

9.3.2. Catalytic activity

HPL is still not registered in the enzyme classification (64), but belongs to the cytochrome P450 enzymes (121). HPL, however, does not need a cofactor such as molecular oxygen which is essential for most P450 enzymes (80). Lyases generally catalyze an elimination reaction in which a double bond is formed, and HPL catalyses the chain cleavage of the fatty acid hydroperoxide to form aldehydes and oxoacids (177). These hydroperoxides origin from LOX reactions, autoxidation or photochemical oxidation (178).

Shibata et al. (156) demonstrated that HPL is a heme protein. Results from green bell pepper by the same authors suggested the involvement of a metal ion in HPL (157), and Kandzia et al. (86) stated that since HPL is a P450 enzyme an iron atom is bound by a heme molecule in the active

site. Noordermeer et al. (121) proposed a reaction mechanism based on the known chemistry of cytochrome P450 enzymes where the iron atom takes part. Matsui et al. (110) reported that HPL from tea has a sulfhydryl group in its active center and Wurzenberger & Grosch (190) assumed by experiments with HPL originating from mushroom that the enzyme possesses a hydrophobic pocket in which the hydroperoxide is fixed. **Figure 9.4** summarizes the catalytic activity of HPL.

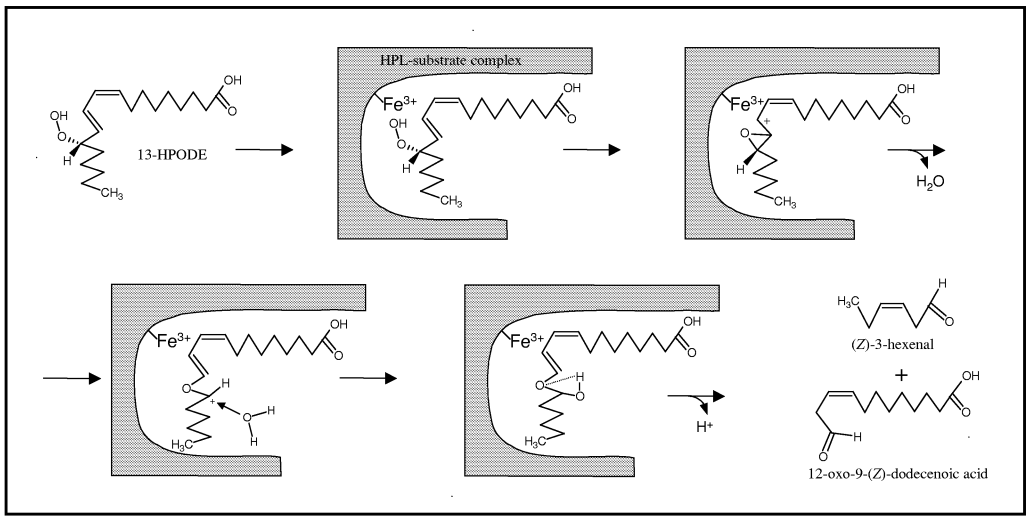

Figure 9.4. Catalytic activity of HPL. 13-HPODE = 13-(*S*)-hydroperoxy-9-(*Z*)-11-(*E*)-octadecadienoic acid. Modified after (71, 121).

HPL catalyses the cleavage of the C-C bond located between the hydroperoxide group and the double bond with an (*E*)-conformation (45, 141) as shown in **Figure 9.4**. First HPL catalyses cyclization of the protonated hydroperoxide to a 12,13-epoxycarbonium ion with loss of a molecule of water. This forms an allylic ether cation with charge located at carbon-13 adjoining the oxygen. Finally, a water molecule is added to the oxygen-stabilized carbonium ion which causes the chain cleavage and yielding the aldehyde and the oxoacid (71).

The product formed is dependent on the hydroperoxide cleaved; most of the commonly produced volatile compounds are outlined in section 8.2 and 8.3.

9.3.3. Substrate specificity

HPL's originating from plants are specific in their cleavage of hydroperoxides, some only act on 9-hydroperoxides, other only on 13-hydroperoxides and some react on both hydroperoxides (175). This specificity may or may not be related to the specific action of LOX. Grechkin (64) stated that the specificity of HPL normally corresponds to the specificity of LOX, which also seems rational, but Sanz et al. (142) asserted that the specificities are not related. Results of investigations on HPL from green bell pepper suggest that the enzyme recognizes the partial structure of the substrate and a terminal carboxylic group. The results also indicate that HPL has a strict substrate-recognition system in terms of geometrical and positional structure (157).

Origin of the HPL is very important in relation to specificity. Investigations on HPL from sunflower showed that leaf and cotyledon HPL utilized the linolenic acid hydroperoxide most readily, while root HPL did not show any preference between hydroperoxides from linoleic acid and linolenic acid (176). HPL in potato tubers only showed activity against the 13-HPOTE and not the 9-HPOTE (173). Only the 13-hydroperoxides of both linoleic acid and linolenic acid were cleaved by tomato HPL with a clear specificity towards the linolenic hydroperoxide. C-9-hydroperoxides were not a substrate (166). Purified tomato HPL converted 13-HPOTE 8 times faster than 13-HPODE (166). Activity of HPL from green bell pepper was about 12 times higher with 13-HPOTE than with 13-HPODE (157). **Paper 5** found no difference in the activity of leek HPL on 9-HPODE and 13-HPODE.

9.3.4. Impact of pH and temperature

The pH optimum ranges from 5.5 for green bell pepper (157) to pH 8 for cucumber cotyledons (111). Activity of purified HPL from spinach leaves decreased sharply at pH below 7 and was still high at pH 9 (178). HPL from germinating watermelon seedlings has a pH optimum of 6-6.5 (177) and pH optimum of HPL from tomato was 6.5 (166). It is not possible to predict the pH optimum of HPL in *Allium* from this information, but the assay described in **paper 5** showed good activity at pH 6.0.

Purified HPL from cucumber showed a total loss of activity when heated to 70°C for 2 minutes while 50°C for 10 minutes resulted in 50% reduction of activity (181). **Paper 6** demonstrated that HPL is more heat stable than LOX, when the heat treatment was applied to the leek slices. Phillips & Galliard (127) on the other hand reported that purified HPL from cucumber was extremely heat labile, and the same was found in soybean seedlings (123) which probably means that HPL is protected in the intact cell.

9.3.5. Natural inhibitors

At high 13-HPOTE concentrations (higher than 0.3 mM) product inhibition of HPL from tomato was observed (166), which was assumed to be due to (*E*)-2-hexenal. α,β-unsaturated aldehydes can react with proteins and in particular sulfhydryl enzymes can be inactivated this way (166). Consequently, 12-oxo-10-(*E*)-dodecenoic acid – one of the products of HPL reaction on 13-HPODE and 13-HPOTE could also have an inhibiting effect. HPL activity in green bell pepper was considerably inhibited by lipophilic antioxidants such as α-tocopherol and nordihydroguaiaretic acid. Reversible inhibition by lipophilic antioxidants indicated that the antioxidant might affect an essential step, probably the radical-formation step (72, 157).

9.3.6. Protein characteristics

HPL is highly hydrophobic which is expected from membrane associated enzymes (178). HPL from *Allium* has not been characterized. The molecular weight of HPL in spinach has been estimated to 220 000, but this was together with hydroperoxide dehydrase (HPD). Both enzymes are hydrophobic and aggregate to form high molecular complexes (178). HPL from germinating

watermelon seedlings has a molecular weight in the excess of 250 000 (177). Enzymatic activity of HPL from green bell pepper fruits was separated into two fractions, and both isoenzymes were trimers with subunit molecular weight of 55 000 and similar enzymatic properties (157). Enzyme extracted from cucumber cotyledons showed two isoenzymes, one cleaving the 9-hydroperoxide and one cleaving the 13-hydroperoxide (111).

9.3.7. Assays

HPL can be assayed direct by following the decrease in absorbance at 234 nm due to loss of conjugated diene structure in the fatty acid hydroperoxide analogous to the assay described for LOX. As mentioned in section 9.2.7 proteins and other compounds present in crude and partially purified plant extracts often contribute to strong absorption at 234 nm and also the presence of HPD will influence the results of HPL as HPD competes for the same substrate (175).

Another possibility is a coupled continuous spectrophotometrical assay. ADH is converting the aldehydes formed by HPL under the consumption of NADH, which is followed by a decrease at 340 nm, where fatty acid hydroperoxides do not absorb significantly (175, 192).

No matter which assay is chosen, the extraction method is very important as the HPL is membrane bound. Enzymatic activity of HPL purified from tomatoes was only stable if Triton X-100 was present (166), and experiments with sunflower HPL showed increased catalytic activity if Triton X-100 was present (176). **Paper 5** also reported that best results of HPL extraction was found when Triton X-100 was added to the extraction buffer.

9.4. Alcohol dehydrogenase

ADH is a very widely distributed enzyme, which has been found in microorganisms, yeast, and animal (36), and a wide variety of higher plants (116). Alcohol dehydrogenase is hardly investigated in leek or any other *Allium* species, and the description of the enzyme is done mostly on the basis of studies on other sources. The purpose of ADH is suggested to be involvement in cell survival in periods of reduced oxygen level, regulation of development of the plant, lignification of cell walls and biogenesis of flavor volatiles (116). The last-mentioned is probably closely related to the finding of highest ADH activity in the full-red stage of maturity of tomatoes (192). ADH of higher plants plays an important role in the energetic metabolism of seeds (28). ADH is essential in the conversion of pyruvate, the product of glycolysis, to ethanol by yeast and other microorganisms, and for that reason ADH is a very important enzyme from a technological point of view.

9.4.1. Localization in the cell

The localization of the enzyme in the cell is determined by the source of the cells. Most bacterial ADH's are located in the cytoplasm although some are membrane bound (36). *Saccharomyces cerevisiae* possess three ADH's, two of them are situated in the cytoplasm and the last one is occurring in the mitochondria (36). ADH from rape is localized in the cytoplasma (160).

9.4.2. Catalytic activity

Alcohol dehydrogenase (EC 1.1.1.1) is an oxidoreductase, which catalyzes the reduction of carbonyl compounds such as aldehydes and ketones and the oxidation of alcohols (36). This process involves a cofactor; NADH or NADPH for the reduction of carbonyl groups and NAD^+ or $NADP^+$ for the oxidation of alcohols. ADH's can be divided into NAD^+ dependent (EC 1.1.1.1) and $NADP^+$ dependent (EC 1.1.1.2) (116). There is little information on the mechanism of action of ADH from higher plants (60).

ADH follows an ordered bi-bi mechanism in which the cofactor must bind before the substrate (27, 161). Some ADH needs zinc to be activated and some require iron to be in the active form. In zinc-containing ADH cysteine acts as the binding site for the catalytic zinc atom (31, 36, 142). The zinc atom binds the substrate and participates in an acid-base system for removal of protons in the horse liver ADH (83), which is one of the best studied ADH (183). The reaction mechanism of ADH is to my knowledge not investigated in *Allium*, but most plant ADH are NAD^+ dependent metalloprotein enzymes (105). Maize ADH is shown to contain zinc, which is essential for catalysis (168).

The mechanism of activity for horse liver ADH is described by (183) (**Figure 9.5**). The enzyme consists of two subunits each with two clearly separated domains. One domain binds the coenzyme (NAD^+ or NADH); the other binds the zinc atom of the subunit. The catalytic sites are in the junctions between the two domains. The active site has a hydrophobic pocket (or barrel) through which the substrate must enter.

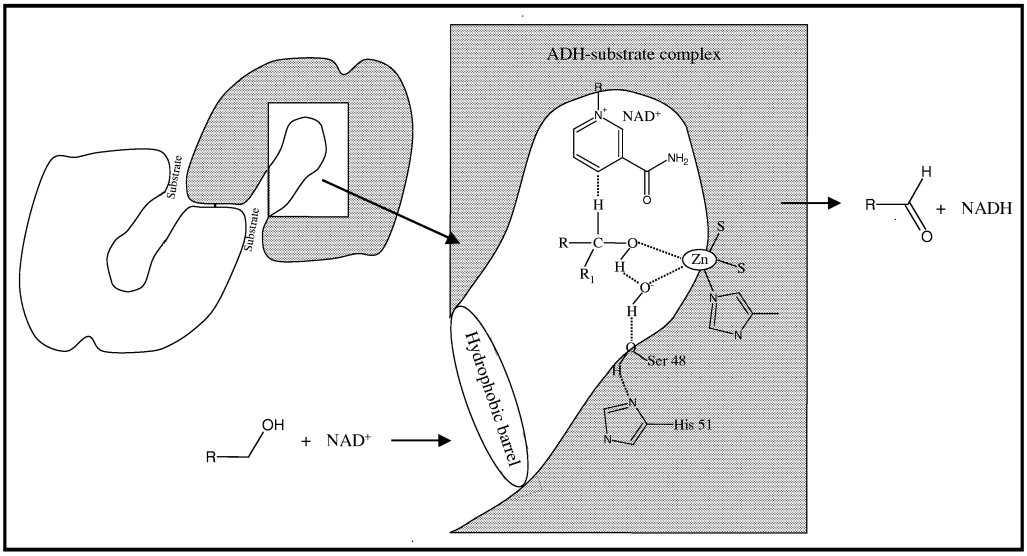

Figure 9.5. Catalytic activity of ADH. Modified after (183).

Alcohol binds directly to zinc as the alcoholate ion via a hydroxide ion on the zinc, which is preformed from water on binding of NAD^+. The zinc bound hydroxide ion is postulated to be the general base catalyst for the oxidation of alcohols and the zinc-bound water molecule as the general acid catalyst for the reduction of aldehydes. Histidine and cysteine are involved in the NAD^+ binding in pea ADH (28).

9.4.3. Substrate specificity

The carbonyl group has to be present as an aldehyde group, ketone functional groups in the compound do not work as effective ADH substrates (175). ADH from rape has a relative broad substrate specificity for alcohols and aldehydes (162). Unsaturated alcohols are oxidized more rapidly by rape ADH than the saturated analogues and aldehydes are better substrates than alcohols (160). ADH from orange and pea reaches highest activity with (*E*)-2-hexenal (31). (*Z*)-3-hexenal is not an effective substrate to yeast ADH (175) but to ADH from other sources (64). As shown in **Figure 8.5**, (*Z*)-3-hexenal can also be converted by an isomerase to form (*E*)-2-hexenal and then be metabolized to (*E*)-2-hexenol.

Rape ADH showed K_m values for aldehydes in the range of 1 - 50 mM, and K_m increases with increasing length of the carbon chain of the aldehydes (160).

9.4.4. Impact of pH and temperature

The catalyzed reaction is highly reversible depending on the pH in the environment of the enzyme. At pH 7 which is close to the pH in the plant, the alcohol formation occurs most readily, whereas at pH 9 the reaction is favored towards aldehyde formation (36). For most ADH the direction of the equilibrium favors alcohol production and NAD^+ (183).

For many ADH the pH optimum depend on the direction of the reaction. For the rape ADH the pH optimum for reduction of aldehyde to alcohol was near 7, while the pH optimum for oxidation of alcohol to aldehyde was 8.5 (183). ADH from rape is most stable at pH 6.5-7 (161).

ADH is denatured by heating above 60°C but the enzyme-NAD complex is thermally more stable than the enzyme alone (161). The analogous was found for LOX (3). For rape ADH the K_m value for ethanol increased with increasing temperature (161).

9.4.5. Natural inhibitors

Propyl 2-propenyl thiosulfinates, which is produced by alliinase in *Allium* species, is found to strongly inhibit alcohol dehydrogenase (4). Investigations both on $NADP^+$ dependent ADH from *Thermoanaerobium brockii* and NAD^+ dependent ADH from horse liver showed irreversible inhibition (133). This investigation showed that 2-propenyl thiosulfinate can be considered as a modulating agent, which regulates enzymatic activity of SH-containing enzymes by thiol-disulfide exchange reactions. But presence of glutathione inside the cell can neutralize the di-2-propenyl thiosulfinate activity in the intact cell.

Rape ADH is inhibited by organic acids e.g. acetic acid, propanoic acid, and butanoic acid (162), and pyridoxal-5'-phosphate, the cofactor of alliinase, is shown to inactivate pea ADH reversible (28). **Paper 3, 5** and **6** did show activity of ADH in crude leek enzyme extract, which indicates that leek ADH is not inactivated by pyridoxal-5'-phosphate.

9.4.6. Protein characteristics

ADH can be divided into different groups depending on the polypeptide chain length. The short chain group consists of non-metalloenzymes with subunits of approximately 250 residues, the medium chain group often contains zinc and with subunits of 350-375 residues, and the long chain group consists of subunits with over 700 residues (36). ADH has probably not been investigated in leek or other *Allium*. In olive fruit three isoenzymes were found, one was NAD dependent and two were NADP dependent (140). Subunits of mushroom ADH showed a size of 40 000 dalton and the enzyme consisted of two subunits (122), like the horse liver ADH illustrated in **Figure 9.5**.

9.4.7. Assays

The common assay is based on a spectrophotometrical measurement of NADH which can be monitored at 340 nm. The assay can either utilize the reduction of NAD^+ under the oxidation of alcohol or the oxidation of NADH under the reduction of aldehyde. Another assay is based on fluorescence spectroscopy measurement. The increase of fluorescence emission due to NADH formation is followed at 450 nm upon excitation at 340 nm. This method should be more sensitive than the spectrophotometrical measurement (36).

10. Factors Affecting the Aroma Composition of Processed Leek

Few investigations are done on the development of aroma compounds in leek or other *Allium* species during processing or frozen storage except for **papers 1**-**4** and **6**-**7**. Consequently, the impact of slice thickness, blanching, or frozen storage on the aroma composition of leek is not examined into details. For that reason general information generated from other crops is cited together with the literature referring to *Allium*.

The determined aroma composition in processed leek is a result of many factors. The potential of producing aroma compounds in the fresh leek, the treatment before storage (slicing, blanching and freezing), the storage time and temperature, the thawing process and last the method of analyzing the aroma content. The first and the last parameter has already been discussed, the processing and storage will be outlined in this chapter.

10.1. Slice thickness

The rate of development of volatile aroma compounds in freshly cut tissue depends on the degree of tissue disruption (16, 39, 92, 112). Increasing the tissue breakdown by cutting thin slices generally increases the total production of volatiles, because the ratio of ruined cells to intact cells increases with thinner slices, and therefore the liberation of enzymes and substrate increases. This is of course important for the instant formed aroma compound, but may also influence the development of aroma compounds during frozen storage.

In the commercial production of frozen leek slices the slicing process can be introduced well ahead of the freezing process. This makes the slicing procedure very important in relation to formation of aroma compounds, as the enzymatic processes can proceed from the moment of slicing, until the temperature in the tissue is low enough to prevent it. The effect of slice thickness on aroma formation and enzyme activity is illustrated in **Table 10.1** both for unblanched and blanched leek slices, the blanching effect is discussed in section 10.2.

Paper 7 reported that the total amount of produced sulfur compounds was almost tripled when the slice thickness was decreased from 15 mm to 4 mm in the unblanched fresh samples (**Table 10.1**). The investigations reported in **paper 3** showed an even more noticeable effect as the production of sulfur compounds in the 4 mm slices was seven times larger than the production in the 15 mm slices. This enhanced production is probably because of liberation of more cysteine sulfoxides from the cytoplasm in the 4 mm slices, but also liberation of more cysteine sulfoxides from the γ-L-glutamyl peptides are a possibility because of additional activity of glutamyl peptidase. The activity of alliinase was influenced in an opposite way (**Table 10.1**) as the 15 mm slices showed more than twice the activity of the 4 mm. This suggests that alliinase maintain stability better in intact tissue.

Table 10.1. Impact of slice thickness on production of aroma compounds and enzyme activity in unblanched (UNB) and blanched (B) fresh leek slices (0 M) and in unblanched and blanched leek slices stored frozen at -20°C for 12 months (12 M). Modified from **paper 7** and (Nielsen, unpublished data).

		4 mm 0 M	**15 mm 0 M**	**4 mm 12 M**	**15 mm 12 M**
Sulfur[a]	UNB	4.90±0.46	1.35±0.45	2.41±0.69	0.656±0.18
	B	2.50±0.24	1.09±0.23	2.19±0.13	1.14±0.090
Aldehydes[b]	UNB	0.694±0.11	0.681±0.21	11.0±0.87	7.86±2.3
	B	0.498±0.18	0.477±0.11	1.67±0.35	2.13±0.40
Alliinase[c]	UNB	0.00487±0.0034	0.0103±0.0033	0.000304±0.000041	0.000581±0.00019
	B	0.00269±0.0010	0.00180±0.00072	0.000439±0.00016	0.000194±0.000071
LOX[c]	UNB	0.00280±0.0010	0.00255±0.00042	0.000135±0.000028	0.000308±0.000095
	B	0.00±0	0.00±0	0.00±0	0.00±0
HPL[c]	UNB	0.00526±0.0027	0.00188±0.0013	0.00550±0.0025	0.000226±0.000064
	B	0.00170±0.00060	0.00278±0.00067	0.00123±0.00030	0.000324±0.000042
ADH[c]	UNB	0.000637±0.00023	0.000574±0.00021	0.000133±0.000056	0.000120±0.000044
	B	0.000362±0.00013	0.000130±0.000014	0.0000487±0.00002	0.0000593±0.00002

[a] Total amount of sulfur compounds in mg/L. [b] Total amount of aldehydes except for propanal, 2-methyl-2-butenal, and 2-methyl-2-pentenal in mg/L. [c] Katal/kg protein.

The effect of slicing thickness on the formation of sulfur compounds was still present after frozen storage for 12 months. The total amount of sulfur compounds diminished by one half in both slice thicknesses, which implies that the ratio between 4 mm and 15 mm was not altered during frozen storage (**paper 7**). This was also found by **paper 3**. Alliinase activity diminished by one half for both slice thicknesses indicating that there was no effect of slice thickness on the freezing stability of alliinase.

The total amount of produced aldehydes in the freshly cut leek was not influenced by slice thickness (**Table 10.1**), even though more ruined cells per weight unit are present in the thin slices. **Paper 3** found a minor but significant increase in aldehyde production in the 4 mm slices compared to the 15 mm slices. The catalytic activity of LOX and ADH was not influenced by slice thickness in the fresh samples whereas the activity of HPL was statistically higher in the thickest slices (**Table 10.1**). The total amount of aldehydes after frozen storage on the other hand was influenced by the slice thickness, as the 4 mm slices produced more aldehydes than the 15 mm slices. This was partly reflected in the enzyme activity as the activity of LOX was highest in the 15 mm slices, the HPL activity was highest in the 4 mm and ADH was not influenced. This effect of slice thickness on aroma formation was also observed in **paper 3**, but only HPL was significantly influenced by slice thickness; 15 mm gave the highest activity after 12 months of frozen storage.

These results all together signify that the slice thickness is a very important parameter in the aroma formation of fresh leek slices and in the effort to keep a high quality of the frozen leek slices, especially when the storage period is long.

10.2. Blanching

Production of frozen vegetables often includes a blanching step mainly to prevent off-flavor formation, as most raw vegetables changes quickly even when stored frozen (186). This blanching process does benefit the product but there are also drawbacks to it (186). The heating process destroys the catalytic activity of enzymes, which will prevent off-flavor formation and enzymatic browning, and kills microorganism. Furthermore, air will be expelled from the tissue; this can reduce autoxidation during storage and stabilize color originating from chlorophyll or carotenoids. But usually there is some loss of texture, loss and/or alteration of flavor, and loss of vitamins during the blanching process and the cooling (9, 95, 131, 186).

In order to retain the product as close to the fresh appearance as possible and at the same time inactivate enzyme, the minimum effectual heat treatment should be applied. Many of the occurring quality changes during frozen storage of vegetables are due to enzymatic activity (7), and therefore it seems reasonable to choose an enzyme as indicator of adequate blanching. Hildebrand (74) stated that the destruction of LOX activity is one of the principal reasons for the need for blanching of food products prior to freezing. Barrett and Theerakulkait (9) suggested that LOX is used as an indicator enzyme of sufficient blanching instead of peroxidase or catalase, which are commonly used. This is because LOX is widely distributed in vegetables, the enzyme is involved in off-flavor formation during storage, and it is heat labile, which ensures that overblanching does not occur.

Blanching experiments on leek slices showed that LOX is more heat labile than HPL, ADH and alliinase, and the aroma analyses showed that inactivation of LOX is effective in the restricting of off-flavor formation during frozen storage even though HPL and ADH was not totally inactivated (**paper 6**). **Table 10.1** shows the activity of alliinase, LOX, HPL and ADH right after the blanching procedure. Blanching time was adjusted so that LOX was inactivated in both slice thicknesses, and this also showed that none of the other three enzymes were totally inactivated. The activity of these enzymes was decreasing during frozen storage regardless of slice thickness, but still present after one year of storage.

The blanching process is either an immersion of the vegetables to hot water or a steam treatment. In both cases leaching of vitamins, carbohydrates, aroma compounds, and other water-soluble components to the blanching water happens. This is most distinct for the water blanching, as the steam blanching uses less water (131). **Paper 2** showed a considerable loss of sulfur compounds to the blanching water during blanching of 15 mm leek slices. If water cooling is applied after the steam blanching this difference is minimized. Cooling with cold air or spraying of cold water reduces the leaching but results in weight losses (126).

Loss of flavor is a serious disadvantage of blanching. Investigations with blanching of carrots showed that nearly all the present volatile compounds decreased with blanching time and a loss of 70% of the total volatile content was measured after 1 minute of blanching (152). As the

flavor of freshly cut *Allium* species is characterized by heat labile thiosulfinates, the blanching process will most likely alter the aroma profile of leek. Boiled onion flavor comes from propyl- and propenyl- di and trisulfides (16). **Paper 2** displayed an increase of sulfides during blanching times up to 2 minutes, and beyond that a decrease was observed, while aldehydes decreased effectually by increased blanching time. Consequently, blanching time should be as short as possible.

Tokitomo (170) found that thiosulfinates were not detectable in cooked onions, but that trisulfides such as dimethyl trisulfide, methyl propyl trisulfide and dipropyl trisulfide in onions increased by heating, and propenethiol and 2-methyl-2-pentenal decreased. The pyruvate value, which is a measurement of the pungency of the vegetable, because pyruvate is closely related to the formation of thiopropanal-*S*-oxide, was by blanching of onions decreased to 2.6% of that present in the raw onions. The aroma analysis showed that the major component in the blanched samples was propanethiol (52).

Blanching is not exclusively reducing aroma content though, as some sulfur compounds are generated thermally. 3,5-dimethyl-1,2,4-trithiolane is among others shown to form during the heating of 2-PeCSO (99). Boiled onions also have a tendency to develop a sweet taste, which was found to partly be due to the development of propanethiol, which is 50 – 70 times as sweet as sucrose (100). Boelens et al. (16) compared the aroma of raw and boiled onions and found that although the absolute quantities of dimethyl thiophenes are about the same in both samples their relative importance is much larger in the boiled onions. 2,4-dimethylthiophene and 3,4-dimethylthiophene are described as fried onion (16), or as solvent, plastic-like (56).

The investigation by (170) also showed that slicing of the onions before heat treatment enhanced the formation of these trisulfides effectually compared to whole boiled onions. This increase of trisulfides could be on account of liberation of cysteine sulfoxides from γ-glutamyl peptides, which is probably promoted by heat treatment (196). Investigations on cut green onions also showed higher thiosulfinate concentrations in raw onions when compared to heat-treated onions (77). **Table 10.1** shows the result of an investigation on blanched and unblanched leek slices (**paper 7**). These results support the above mentioned effect of slicing, because 4 mm blanched samples developed more sulfur compounds than 15 mm samples did.

The blanching of the leek slices has an impact on the aroma profile of the frozen stored leek slices. Petersen el al. (125) found that blanching of leek slices prior to frozen storage gave less intense leek flavor but also less development of off-flavor. This is consistent with what was reported by **paper 4, 6** and **7**.

Paper 4 compared the aroma of blanched and unblanched leek slices after a year of frozen storage to the aroma of fresh leek slices by GC-olfactometry. The conclusion regarding the effect of blanching prior to frozen storage was that blanching to some degree prevents changes in aroma profile, but also reduces the perceived intensity of the aroma compounds. When looking

at the most important compounds determined by GC-O, the fresh samples and the frozen stored blanched samples had the same compounds in the first, third and fifth place, whereas the unblanched frozen samples had none of the compounds in the same position as the fresh samples (**paper 4**, table 2).

10.3. Frozen storage

The freezing process is said to be one of the best methods available in the food industry for maintaining high quality of food products. The quality of frozen vegetables is influenced though of some of the consequences of freezing, e.g. destruction of cytoplasmic structure, loss of turgor pressure, and weakening of the cell wall (132). This will affect texture and aroma formation in the product. It is essential for a good vegetable quality during freezing that the time between harvest and processing is short, and that the product is frozen rapidly (95).

10.3.1. The freezing process

During freezing pure water will separate and freeze first. This causes a concentration of solutes such as enzymes and salts, and also considerable changes in pH and ionic strength in the micro-environment of the enzymes can occur (179). It is well known that slow freezing induces the formation of large extracellular ice crystals that may cause mechanical damage, and an increased extracellular concentration of solutes, which over time causes cell dehydration and death through osmotic plasmolysis and membrane damage (32), while rapid freezing enhances formation of many smaller ice crystals (67).

The actual freezing process can possibly alter the aroma profile of vegetables by affecting the catalytic activity of enzymes. **Table 10.2** shows the activity of alliinase, LOX, HPL and ADH measured in fresh leek slices and in frozen leek slices one day after freezing (Nielsen, unpublished data). The crude enzyme extract was made directly of the frozen slices so that no thawing was applied. The activity of alliinase was almost halved (not significant on 5% level) by freezing in 4 mm slices, while it was not affected by freezing when present in 15 mm slices. This indicates that alliinase to some degree is protected in the intact tissue.

Table 10.2. Catalytic activity of alliinase, LOX, HPL, and ADH in leek slices measured in fresh leek slices and in frozen leek slices one day after freezing. (Nielsen, unpublished data).

	4 mm slices		15 mm slices	
	Fresh	**Frozen**	**Fresh**	**Frozen**
Alliinase	0.00487±0.0034	0.00281±0.0011	0.0103±0.0033	0.00914±0.0074
LOX	0.00280±0.0010	0.00321±0.0027	0.00255±0.00042	0.00215±0.0014
HPL	0.00526±0.0027	0.00221±0.00064	0.00188±0.0013	0.00565±0.0029
ADH	0.000637±0.00023	0.000633±0.00025	0.000574±0.00021	0.000340±0.00015

There was no statistical effect of the freezing on LOX and ADH, whereas the activity of HPL decreased by freezing in the 4 mm slices and increased (not significantly) in the 15 mm slices.

Freezing stability of ADH was influenced by slice thickness; the 4 mm frozen slices gave significantly higher activity than the 15 mm frozen slices. No information has been found about stability of LOX or ADH during the freezing process, but the freeze-thawing process gave rise to a minor loss of activity (10%) for crude tomato HPL (166). Freezing at -25°C and thawing the next day of onion caused large losses of pungency (78), which could be due to loss of alliinase activity.

Wäfler et al. (179) demonstrated that the effect of freezing on activity of alliinase was depending on the freezing technique and the thawing procedure. In their work the enzyme was not denatured by the actual freezing, but by the cellular alterations that will occur during slow freezing and thawing of the tissue. Slow freezing leads to formation of ice crystals in the tissue, which ruins cell compartmentation and make proteolytic degradation of cell substance possible. The discovered loss in alliinase activity was proved not to be a result of loss in alliinase protein. It was suggested that the freezing induces conformational changes in the alliinase protein. There are hydrophobic areas located at the surface of the enzyme, which cause an ordered structure of the surrounding water molecules. This structure can be disorganized by freezing water surrounding the hydrophobic parts, possibly leading to aggregation and precipitation.

10.3.2. Thawing of frozen tissue

Tissue damage is introduced in the freezing process because of the expansion of water (107). This becomes crucial during the thawing process of unblanched products as the compartmentation of enzymes and substrate is ruined. As the temperature rises, the enzymatic processes can proceed unhampered, if the enzymes were not inactivated by the freezing process and/or the frozen storage. Upon thawing extracellular ice does not re-enter the cells, and this may cause extensive drip loss, texture softening and closer enzyme to substrate interactions (32).

An investigation on diced onions (149) showed that frozen thawed onion dices were not as turgid as freshly diced onions, probably because of loss of water. Ice crystals grow in size during frozen storage by recrystallization, and this is depending on the storage time and temperature. The shape of the ice crystals becomes rounder during this recrystallization process, and this is important in the thawing process, because the shape might be related to the water-holding capacity of the food during thawing (67).

When frozen leek slices are thawed, it is usually in connection with a heat treatment, which means that the increasing temperature in the tissue will accelerate enzymatic processes until the temperature of inactivation is reached.

10.3.3. Storage atmosphere

The impact of storage atmosphere on the alterations of aroma profile of frozen leek slices would be expected to be greatest on the LOX pathway produced aroma compounds, as oxygen takes part in the LOX catalyzed reactions. Alliinase is not dependent on oxygen for the catalytic activity, and therefore there is no obvious reason to believe that the atmosphere would affect the

activity of alliinase during storage, unless liberation of precursors more readily occurs if oxygen is present. Still Uddin & MacTavish (171) observed an increase in catalytic activity of alliinase in onion bulbs stored at 0.5°C in atmospheric air whereas storage in 2% O_2 and 2% CO_2 gave a reduction in activity when compared to fresh onion. Investigations reported in **paper 6** revealed no differences in activity of alliinase when stored in atmospheric air and in 100% nitrogen for 6 or 12 months.

Paper 3 and **6** showed an effectual impact of storing unblanched frozen leek slices in 100% nitrogen for one year as the total concentration of sulfur compounds after one year is almost the same or even higher than in the fresh leek slices. Unblanched samples stored in atmospheric air lost about half of the total concentration of sulfur compounds. The development of aldehydes was at the same time suppressed effectual in the slices that were kept in 100% nitrogen. Activity of LOX was declining through the storage period but was highest in the leek slices stored in 100% nitrogen. Activity of HPL and ADH was not influenced by the storage atmosphere.

Paper 6 also investigated the influence of storage atmosphere combined with blanching. This proved that blanching of the leek slices was more efficient in restraining the development of off-flavor than the storing of unblanched samples in 100% nitrogen was. However, sulfur compounds were preserved better in the atmosphere of 100% nitrogen. The results also showed that it is possible to obtain both advantages by packaging blanched samples in 100% nitrogen, as this combination kept the initial concentration of sulfur compounds and aldehydes.

Under conditions of oxygen deficiency LOX can produce a number of compounds (fatty acid dimers, oxodienes, and epoxyalcohols) of free radical conversions instead of normally produced hydroperoxides (64). The effect of this on the aroma profile is uncertain, but some of the compounds are probably not volatile. The investigations reported in **paper 3** and **6** did not detect any new compounds in the samples stored in 100 % nitrogen, and the generation of aldehydes is reduced significantly compared to samples stored in atmospheric air.

10.3.4. Storage time

The three investigations on frozen storage of leek slices reported in **paper 1**, **3**, **4** and **6** clearly demonstrate that the aroma composition alters during storage and that the storage time is an important parameter in this alteration. Lipid oxidation is the limiting quality factor in many stored vegetables (9), and when leek slices are stored frozen for a longer period, the production of aldehydes have an influence on the aroma profile, because of the diminishing content of sulfur compounds (125). As seen in **Table 10.1,** aldehydes are accumulating during frozen storage, and they are most likely appearing as off-flavors when present in larger amounts (124, 125).

Paper 4 showed by GC-O analysis that most of the present sulfur compounds decreased in intensity after frozen storage and that most aldehydes increased in intensity. The sulfur compounds were described as fresh leek or strong raw onion, and a few as rotten. The aldehydes

detected by GC-O were mostly described as unpleasant (e.g. rotten, rubber, paint, sweat, or compost) and only (E)-2-hexenal was described as fresh onion.

Paper 1, **3** and **6** demonstrated that sulfur compounds decrease during frozen storage and that aldehydes and other compounds increase during frozen storage. Baardseth (7) on the contrary found that unblanched leek did not develop detectable off-flavor, and no changes in total lipid content were detected after the storage. This is in contrast to most other investigations but could be explained by a short storage time. The aroma of unblanched frozen leek slices was found to be constant for approximately 2 months by Petersen et al. (125). After that period the aroma profile changed towards more aldehydes and less sulfur compounds. **Paper 1** and **3** showed likewise that the alterations of aroma composition primarily occur after 2-4 months of frozen storage.

Freezing of chives changed the headspace profile by decreasing the formation of volatile sulfur compounds and by increasing the five- and six-carbon aldehydes (85). Leino (106) reported a decrease in the relative proportion of disulfides in frozen samples of cut chives compared to fresh samples, and freezing also altered the perceived intensities of odor attributes determined by sensory analysis. Freezing of onions was found to give the same characteristic taste as fresh onion but milder (52).

The reason for this alteration of the aroma composition in frozen stored *Allium* species is probably alterations of activities of the enzymes that catalyse formation of aroma compounds, or alterations in accessibility of the substrates. As explained in section 10.3.1 and 10.3.2 the freezing and frozen storage can liberate substrates because of destruction of cell structure, and if the enzymes are not denatured by freezing, reactions can occur during the frozen storage to a minor degree and during the thawing process.

Schwimmer & Guadagni (149) discovered that unblanched onion loose their pungent flavor during frozen storage and at the same time the catalytic activity of alliinase is lost. They demonstrated that this was due to very low enzyme content whereas the onion still contained considerable amounts of cysteine sulfoxides. This indicates that alliinase is denatured during freezing. Kallio et al. (85) stated that alliinase from different *Allium* species is very sensitive to various processing treatments, and that freezing decreases the content of sulfur compounds in the headspace of chives. Frozen storage of whole onion bulbs showed that the content of saturated di- and trisulfides decreased, and that this decrease was dependent on the storage temperature. -18°C and -6°C was tested and -18°C gave by far the largest decrease, and this was interpreted as a proof of irreversible deactivation of alliinase (115).

Freeman & Whenham (54) detected intact activity of alliinase during 30 days of frozen storage (-20°C) of whole onion bulbs, and Lohmüller et al. (108) discovered an increased activity of about 185% after 80 days frozen storage (-20°C) of purified leek alliinase in 10% glycerol, 20 µM P-5'-P and 0.15 M NaCl. **Paper 6** clearly showed that alliinase activity was declining during

frozen storage for one year. The results demonstrated that it is not only the actual freezing process but also the length of the frozen storage that causes the decline as the activity after 6 months was higher than the activity after 12 months. This is consistent with the demonstrated decrease in sulfur compounds during frozen storage.

Paper 1, **3** and **6** demonstrated that the activity of LOX, HPL and ADH was declining over the whole storage period, but while the activity of HPL and ADH is diminutive after one year of frozen storage, the activity of LOX is still approximately 25% of the initial activity. Comparison of aroma content in blanched and unblanched leek slices after frozen storage for one year showed that unblanched samples produce almost four times more aldehydes than the blanched samples (**paper 6**). This indicates that the alterations in aroma composition mainly are caused by enzymatic catalysis despite the observed declining activity. This is probably possible because of increased liberation of fatty acids from cell membranes when cell structure is lost during the frozen storage.

11. Conclusion

The present Ph.D. thesis studied the alterations of enzyme activity and aroma composition that happen during frozen storage of leek slices. These alterations were influenced by storage time, slice thickness, blanching, and storage atmosphere. All of the experimental work demonstrated a pronounced effect of storage time, when frozen storage exceeding 2-4 months. The aroma of the fresh leek slices was characterized by high concentrations of sulfur compounds and low concentrations of aldehydes and other compounds. The total content of sulfur compounds decreased during long-term frozen storage and the total content of aldehydes increased effectual when storing leek slices in atmospheric air. Blanching and packaging in 100% nitrogen influenced this development.

Enzyme activity was effectually influenced by the storage time. All four enzymes investigated decreased during frozen storage and for alliinase, hydroperoxide lyase and alcohol dehydrogenase activity was diminutive after one year. The results demonstrated that it was not only the actual freezing but also the length of the frozen storage that caused the decline of activity. LOX activity was reduced to ~25% of the initial after frozen storage for one year. The aroma analyses demonstrated that production of aldehydes was possible on the basis of the reduced enzyme activity, probably because of increased liberation of substrate due to loss of cell structure during the frozen storage.

Slice thickness is very important for the quality of frozen long-term stored leek slices, because all though thinner slices produces more sulfur compounds both in the fresh leek and in the stored leek, the development of aldehydes are also more pronounced in the thinner slices. The catalytic activity of enzymes is only to a minor degree influenced by slice thickness during frozen storage.

The total concentration of sulfur compounds diminished mostly in the unblanched samples during frozen storage. Blanching prior to frozen storage prevented formation of aldehydes during the storage which indicates that these compounds originate from enzymatic processes rather than autoxidation, because unblanched samples produced almost four times higher concentrations of aldehydes than blanched samples did. A model experiment with crude leek enzyme and unsaturated fatty acids demonstrated enzymatic formation of several of the detected aldehydes and alcohols.

The blanching experiments showed that LOX was the most heat labile enzyme of the four investigated, and HPL the most heat stabile enzyme.

Packaging in 100% nitrogen had a positive influence on the keeping of the initial concentration of sulfur compounds, and did to some degree prevent formation of aldehydes. The activity of LOX was highest in the leek slices stored in 100% nitrogen while the three other enzymes were not influenced by storage atmosphere. The high concentration of sulfur compounds after frozen

storage for one year in the slices packed in 100% nitrogen together with the diminishing activity of alliinase indicate better conservation of the precursors in 100% nitrogen or increased liberation of bound precursors, which the remaining alliinase activity can act upon.

A combination of blanching and 100% nitrogen in the packaging atmosphere of 15 mm leek slices was shown to almost maintain the aroma profile of the fresh leek slices after one year of frozen storage. Alteration of the packaging atmosphere from atmospheric air to 100% nitrogen even without blanching would benefit the quality of the leek slices during frozen storage.

GC-olfactometry analyses showed that there is no single key impact compound in leek aroma. The most important odor active compounds in fresh leek were dipropyl disulfide, methyl 1-propenyl disulfide, pentanal, decanal and propyl 1-propenyl disulfide in order of priority. This altered during frozen storage of unblanched leek slices to pentanal, decanal, 2,5-dimethyl furan, an unknown compound and dipropyl disulfide. Blanching to some degree maintained the order of odor active compounds found in the fresh leek slices but the intensity was diminished.

12. Literature

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13. Publications

Paper 1

Nielsen G. S.; Larsen L. M.; Poll L. Formation of aroma compounds and lipoxygenase (EC 1.13.11.12) activity in unblanched leek *(Allium ampeloprasum* Var. *Bulga*) slices during long-term frozen storage.

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Formation of Aroma Compounds and Lipoxygenase (EC 1.13.11.12) Activity in Unblanched Leek (*Allium ampeloprasum* Var. *Bulga*) Slices during Long-Term Frozen Storage

GHITA STUDSGAARD NIELSEN,† LONE MELCHIOR LARSEN,§ AND LEIF POLL*,†

Department of Dairy and Food Science and LMC, Centre of Advanced Food Studies, The Royal Veterinary and Agricultural University, Rolighedsvej 30, 1958 Frederiksberg C, Denmark, and Chemistry Department, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark

The content of aroma compounds (dynamic headspace) and catalytic activity of lipoxygenase (LOX) (EC. 1.13.11.12) were analyzed in 15 mm unblanched leek slices seven times during 12 months of frozen storage. The aroma profile changed from consisting of almost only sulfur compounds such as dipropyl disulfide [concentration in fresh leek (FL) = 0.197 mg/L, concentration after 12 months of frozen storage (12M) = 0.0409 mg/L] and propyl (*E*)-propenyl disulfide (FL = 0.0437 mg/L, 12M = 0.00452 mg/L) in the fresh leeks to being dominated by numerous saturated and unsaturated aldehydes, such as hexanal (FL = 1.53 mg/L, 12M = 3.63 mg/L), (*E*,*E*)-2,4-nonadienal (FL = 0.000 mg/L, 12M = 0.0647 mg/L), and (*E*,*E*)-2,4-decadienal (FL = 0.129 mg/L, 12M = 0.594 mg/L) at the end of the storage period. The catalytic activity of LOX diminished throughout frozen storage, but ~25% of the original activity was present after 12 months of storage.

KEYWORDS: Leek; flavor; off-flavor; lipoxygenase; frozen storage; dynamic headspace

INTRODUCTION

Commercially produced frozen leek slices are often stored for 1 year or longer, which makes it relevant to investigate the impact of this storage on the development of aroma compounds in the leek slices. Manufacturing of frozen vegetables normally includes a blanching step to destroy the catalytic activity of enzymes and prevent off-flavor formation. Leeks, however, are often processed without blanching, mainly because this has an undesirable effect on the texture. Consequently, enzymes are still present during frozen storage.

The aroma of freshly cut leek and other *Allium* species is dominated by numerous sulfur-containing volatile compounds originating from the decomposition of the odorless nonvolatile precursors *S*-alk(en)ylcysteine sulfoxides by the action of alliinase (EC 4.4.1.4) (*1*, *2*), as shown in **Figure 1**, Scheme 1. Because of compartmentation of alliinase in the vacuole and the cysteine sulfoxides in the cytoplasm (*2*, *3*) volatiles are not produced until cell rupture, for example, by cutting into slices. The products are pyruvate, ammonia, and various sulfenic acids depending upon which radical is present; the ones of common occurrence (*1*) are listed in **Figure 1**. These sulfenic acids are highly reactive (*4*) and will quickly combine to form thiosulfinates (**Figure 1**, Scheme 2). Thiosulfinates are responsible for the odor of freshly cut leeks (*4*, *5*), but as they are relatively unstable (*1*, *6*), they will rearrange to form polysulfides and thiosulfonates (**Figure 1**, Scheme 3). Thiosulfonates are expelling sulfur dioxide to yield the corresponding monosulfide, and the final products of the reaction will end up being a combination of mono- and polysulfides with all of the possible combinations of the radicals listed in **Figure 1**. If the radical on the cysteine sulfoxide is (*E*)-1-propenyl-, thiopropanal-*S*-oxide (the lachrymatory factor) is formed almost exclusively (*7*) (**Figure 1**, Scheme 4). This compound is also unstable and will lead to the formation of propanal and 2-methyl-2-pentenal (*6*).

Another pathway of producing aroma compounds is initiated by the lipoxygenase (EC 1.13.11.12) (LOX)-catalyzed oxidation of polyunsaturated fatty acids with a *cis*,*cis*-pentadiene moiety. This leads to the formation of mainly aldehydes and alcohols, which could contribute to the aroma of fresh leeks but will probably appear as off-flavors when present in larger amounts (*8*, *9*). This is not very pronounced in the fresh leek because of the pungency of thiosulfinates and thiopropanal-*S*-oxide (*6*), although (*E*)-2-hexenal is reported to be a predominant peak in leek aroma (*4*). However, when leek slices are stored frozen for a longer period, the production of aldehydes will have an influence on the aroma profile as well, because of the diminishing content of sulfur compounds (*9*). The aroma compounds actually produced depend on in which position LOX acts on

* Corresponding author (telephone +45-35283240; fax +45-35283265; e-mail lep@kvl.dk).
† Department of Dairy and Food Science.
§ Chemistry Department.

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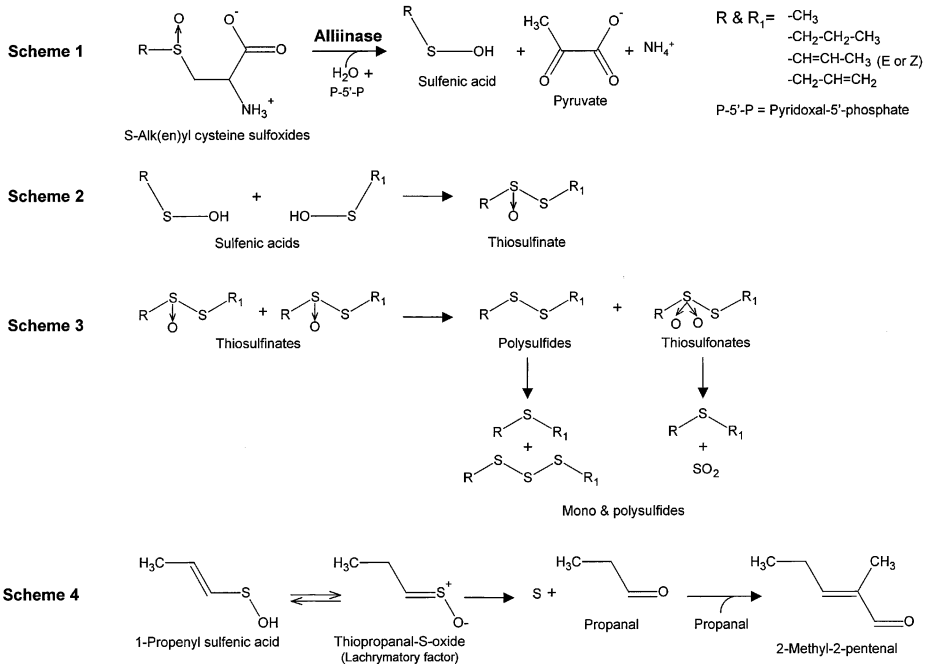

Figure 1. Formation of sulfur-containing aroma compounds in leek and other *Allium* species.

the fatty acid and on the catalytic activities of hydroperoxide lyase and alcohol dehydrogenase. These compounds derived from fatty acids can also be generated by autoxidation during the storage period (*10*), and both pathways will result in accumulation of the products in the tissue.

Frozen leek slices are a commercial product in Europe, but hardly any investigations have been carried out on the changes in the aroma profile during freezing and frozen storage. The objective of the present study was to investigate the development of aroma compounds in leek slices during a long-term frozen storage. Furthermore, an examination of the catalytic activity of lipoxygenase in leek was included to correlate this activity with the development of aroma compounds originating from LOX-catalyzed oxidation of fatty acids.

MATERIALS AND METHODS

Plant Material. Leeks (*Allium ampeloprasum* var. *Bulga*) were harvested fully matured at Funen, Denmark, in October 1999 and immediately after used for the experiment.

Chemicals. All chemicals were of analytical grade and were bought commercially from Sigma-Aldrich.

Sample Preparation. The white part of the leek stem was cut into 15 mm slices and frozen immediately after in a blast freezer at −20 °C for 10 min, so that the center temperature reaches −20 °C. The slices were packed in airtight glass jars with atmospheric air as headspace and kept at −20 °C in the dark until analysis.

Experimental Design. Duplicates of samples were analyzed at harvest time and subsequently after 1, 2, 4, 6, 9, and 12 months of storage. Each sample was analyzed for catalytic activity of lipoxygenase and content of aroma compounds in three replicates.

Lipoxygenase Assay. Enzyme extract was made by mixing 50 g of frozen leek slices with 50 mL of phosphate buffer (0.2 M, pH 6.0) for 45 s in a Waring commercial blender. The slurry was kept on ice with agitation for 30 min (100 rpm), afterward filtered through a paper filter, and kept on ice until time of analysis. LOX was assayed by measuring consumption of initial dioxygen by a Clark electrode (Digital Oxygen System model 10, Rank Brothers Ltd., Cambridge, U.K.) situated in a thermostatic cell (30 °C) with a magnetic stirrer using linoleic acid as the other substrate. Calibration was done at 30 °C by air-saturated phosphate buffer (0.2 M, pH 6.0) (21% dissolved oxygen) followed by the addition of sodium dithionite (0% dissolved oxygen). To determine lipoxygenase activity, 3.00 mL of air-saturated, tempered phosphate buffer was added to the thermostatic cell. After 30 s, 0.40 mL of enzyme extract was added, after which the lid was closed. At 80 s the reaction was initiated by adding 0.20 mL of sodium linoleate solution (10 mM) using a syringe. Dioxygen was monitored over 20 min by measuring the oxygen content every second.

Calculation of Activity. LOX activity was calculated as katals per kilogram of protein under the assumption that air-saturated phosphate buffer (0.2 M, pH 6) at 30 °C with a salt strength of 12.40 g/L contains 7.11 mg of O_2/L (*11*). To determine the activity, the oxygen level (millimoles of O_2) was plotted against time, and the steepest slope (millimoles of O_2 per second) on the curve was found using continuous linear regression over 20 s at a time. Katals per kilogram was calculated as moles of O_2 per second per kilogram of protein. Time elapsed from adding substrate to occurrence of the steepest slope was registered as the length of the lag phase (seconds).

Determination of Protein Content. Protein content in the enzyme extraction was determined by Coomassie brilliant blue according to the method described in US/EG Bulletin 1069 from Bio-Rad Life Science Group, Hercules, CA, using lyophilized bovine serum albumin as the standard protein.

Dynamic Headspace Analysis. Aroma compounds were isolated by dynamic headspace with nitrogen (purity = 99.8%) as purge gas. Frozen leek slices (100 g) were crushed with 150 mL of tap water and 2 mL of internal standard (50 ppm of 4-methyl-1-pentanol) for 90 s in a Waring commercial blender. After the mixture had been transferred to a 1 L glass flask by adding another 150 mL of tap water, it was left for 10 min at 30 °C with agitation (200 rpm) to equilibrate the temperature before purging. Aroma compounds were trapped on 100 mg of Tenax GR (mesh size = 60/80, Buchem bv, Apeldoorn, The Netherlands) for 60 min with a nitrogen flow of 200 mL/min.

Desorption of aroma compounds was done thermally by a short-path thermal desorber (model TD-4, Scientific Instrument Services Inc., Ringoes, NJ). Desorption temperature was 250 °C for 3 min with a helium flow of 11 mL/min. Separation was performed by a GC-MS (HP G1800 A GCD system) with the following conditions: column, DB-Wax from J&W Scientific (30 m × 0.25 mm i.d. × 0.25 μm film thickness); carrier gas, helium; flow, 1 mL/min (constant); split ratio, 1:10; start pressure, 53 kPa; oven program, 40 °C for 10 min, 6 °C/min to 240 °C, constant at 240 °C for 30 min. The mass selective detector was operated in the electron ionization mode, and the *m/z* (mass/charge) ratio ranged between 20 and 450. Identification was done by probability-based matching with mass spectra in the G1035A Wiley library (Hewlett-Packard). Specification of the similarity between mass spectra of the unknown compound and the reference library compound is given as the quality. Values of 90 and above are reliable identifications. Identity was confirmed by checking with mass spectra and retention indices obtained in the laboratory from reference compounds for the following compounds: dimethyl disulfide, methyl propyl disulfide, dipropyl disulfide, dimethyl trisulfide, pentanal, hexanal, heptanal, octanal, nonanal, (*E*)-2-butenal, (*E*)-2-pentenal, (*E*)-2-hexenal, (*E*)-2-heptenal, (*E*)-2-octenal, (*E*)-2-nonenal, (*E*)-2-decenal, 2-methyl-(*E*)-2-butenal, 2-methyl-(*E*)-2-pentenal, (*E,Z*)-2,4-heptadienal, (*E,E*)-2,4-heptadienal, (*E,Z*)-2,4-nonadienal, (*E,E*)-2,4-nonadienal, (*E,Z*)-2,4-decadienal, (*E,E*)-2,4-decadienal, 3-octanone, β-ionone, 1-pentanol, 1-hexanol, 1-octen-3-ol, and 2-pentylfuran.

Quantification. The reference compounds dimethyl disulfide, methyl propyl disulfide, dipropyl disulfide, dimethyl trisulfide, pentanal, hexanal, heptanal, octanal, nonanal, (*E*)-2-butenal, (*E*)-2-pentenal, (*E*)-2-hexenal, (*E*)-2-heptenal, (*E*)-2-octenal, (*E*)-2-nonenal, 2-methyl-(*E*)-2-butenal, 2-methyl-(*E*)-2-pentenal, (*E,Z*)-2,4-heptadienal, (*E,E*)-2,4-heptadienal, (*E,Z*)-2,4-nonadienal, (*E,E*)-2,4-nonadienal, (*E,Z*)-2,4-decadienal, (*E,E*)-2,4-decadienal, 3-octanone, 1-pentanol, 1-hexanol, and 1-octen-3-ol were each dissolved in 400 mL of water and 1.3% sugar added, which equals the sugar content of leeks. Dynamic headspace analysis was performed on the solutions with the same flow, time, and temperature conditions as applied to the leek samples. Each reference compound was analyzed in triplicate in three concentrations. The obtained peak areas were used to calculate the concentration of the compounds in the leeks.

The retention time indices (RTI) of the volatile compounds were calculated with a mixture of hydrocarbons (C_9−C_{26}) as references according to the method of ref *12*.

Statistical Analysis. Analysis of variance was carried out on each of the aroma compounds using the one-way ANOVA procedure in the Analyst part of SAS, version 8.2, SAS Institute Inc., Cary, NC. The effect of the storage period was tested. Sample means were compared by Duncan's multiple-range test. A significance level of 5% was applied.

RESULTS AND DISCUSSION

Table 1 displays all of the aroma compounds identified in fresh leek and in leek slices stored frozen for 12 months. The table shows that the composition of aroma compounds changes from consisting of almost only sulfur compounds to being dominated by aldehydes, which are considered to be off-flavors in larger amounts (*8*, *9*). A total of 14 sulfur compounds were detected, which all more or less show the same pattern. Almost all of them decrease effectively during storage (**Figure 2** displays the development of selected compounds and **Table 2** displays the statistical differences), although some, for example, propyl (*E*)-propenyl trisulfide and dipropyl disulfide, increase during the first 2 months, which is in agreement with the study of ref *9*. 2-Methyl-2-pentenal, which is one of the breakdown products of thiopropanal-*S*-oxide (the lachrymatory factor) (*4*), shows the same development. Others, for example, methyl 2-propenyl disulfide and dimethyl disulfide, are not present at all after 4−6 months of storage (data not shown), whereas

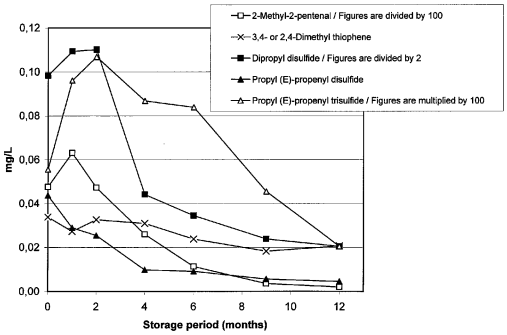

Figure 2. Development of selected sulfur compounds and 2-methyl-2-pentenal in leek slices during frozen storage. See **Table 2** for specification of statistical differences.

dimethylthiophene shows a very limited decrease throughout storage. When the concentrations of, for instance, dipropyl disulfide [fresh leek (FL) = 0.197 mg/L; leek stored frozen for 12 months (12M) = 0.0409 mg/L] and propyl (*E*)-propenyl disulfide (FL = 0.0437 mg/L, 12M = 0.00452 mg/L) in the fresh leek and after 12 months of frozen storage are compared with the threshold value [dipropyl disulfide = 0.0032 ppm and propyl (*E*)-propenyl disulfide = 0.0022 ppm (*13*)], the decrease is very likely to have an influence on the aroma profile of the frozen leek slices.

The production of these sulfur compounds is initiated by the action of alliinase (EC 4.4.1.4) upon *S*-(+)-alk(en)yl-L-cysteine sulfoxide, when cells are crushed. This means that the possibility of producing more sulfur compounds during the headspace analysis is present, when the leek slices are blended in water. This implies that alliinase is able to maintain catalytic activity after frozen storage, which very much depends on the treatment prior to the headspace analysis according to ref *3*. They found that activity was lost after slow freezing or thawing. This is not the case in this work, because the leek slices were quickly frozen in a blast freezer and blended frozen prior to the headspace analysis. Despite this, the present results indicate that either alliinase is not able to retain activity after a frozen storage or the substrate is not present in the right structure any more, because total content of sulfur compounds decreases from 0.544 to 0.110 mg/L.

No thiosulfinates were detected, although they most likely are present in the freshly cut leek slices (*4*). Considering our headspace and GC technique, this was expected, as the thiosulfinates are unstable. They will rearrange and dissociate both over time and by thermal exposure (*1*, *4*, *14*−*16*), and for that reason they will convert into mono- and polysulfides during the dynamic headspace collection and the GC-analysis. Thiosulfinates formed during processing and storage of the leeks will probably also decompose and will therefore not be present in the tissue at time of analysis.

Concentrations of saturated aldehydes (**Figure 3**) showed a substantial increase over the 12 month storage. Hexanal is the only one present in the fresh leek, whereas they all develop during storage. When the concentrations of the aroma compounds are compared with the odor thresholds [0.022 ppm for pentanal and 0.0025 ppm for hexanal (*17*)], there is no doubt that the storage period has an influence on the aroma profile. Hexanal is present at ~1700 times the odor threshold after 12 months, whereas the amount of pentanal at that time is ~20

Table 1. Aroma Compounds Found in Fresh Leek and in Leeks Frozen for 12 Months

compound	quality[a]	RTI[b]	fresh leek, mg/L ± SD[c]	12 month storage, mg/L ± SD[c]	compound previously reported by[d]
sulfur compounds					
methyl pentyl sulfide[f]	96	1140	0.00 ± 0	0.000757 ± 0.00029	
dimethyl disulfide	97	1086	0.0232 ± 0.0062	0.00 ± 0	6,* 21, 22, 23, 24, 25, 26, 27, 28
methyl propyl disulfide	94	1242	0.0729 ± 0.034	0.00728 ± 0.0044	6,* 9,* 29,* 21, 22, 23, 24, 26, 27, 28, 30
methyl 2-propenyl disulfide[f]	90	1296	0.0000819 ± 0.000019	0.00 ± 0	6,* 29,* 21, 23, 24, 26, 27, 30
methyl (*E*)-propenyl disulfide[f]	97	1292	0.0662 ± 0.026	0.00205 ± 0.00090	6*, 9*, 21, 22, 23, 24, 25, 26, 27, 28
ethyl 1-methylethyl disulfide[f]	95	1319	0.000452 ± 0.00019	0.00 ± 0	24
dipropyl disulfide	94	1387	0.197 ± 0.072	0.0409 ± 0.016	6,* 9,* 29,* 21, 22, 23, 24, 25, 26, 27, 28, 30
propyl (*E*)-propenyl disulfide[f]	90	1438	0.0437 ± 0.019	0.00452 ± 0.0021	6,* 9,* 21, 22, 23, 24, 25, 26, 28, 30
dimethyl trisulfide	97	1376	0.0432 ± 0.025	0.000296 ± 0.00014	6,* 9,* 21, 23, 24, 25, 26, 27, 28
diisopropyl trisulfide[f]	90	1656	0.0601 ± 0.033	0.0323 ± 0.014	21
propyl (*E*)-propenyl trisulfide[f]	95	1770	0.000555 ± 0.000080	0.000204 ± 0.000082	6,* 9,* 21, 23, 24, 25, 26, 27
3,4- or 2,4-dimethylthiophene[f]	97	1197	0.0338 ± 0.0072	0.0206 ± 0.0064	6,* 22, 24, 25, 28, 31*
propyl thioacetate[f]	94	1200	0.000886 ± 0.00014	0.00 ± 0	23, 26
3,5-diethyl-1,2,4-trithiolane[f]	95	1751	0.00231 ± 0.00030	0.000873 ± 0.00030	9,* 27
aldehydes					
pentanal	91	984	0.00 ± 0	0.330 ± 0.055	9*
hexanal	97	1111	1.53 ± 0.56	3.63 ± 0.61	9*, 26
heptanal	97	1197	0.00 ± 0	0.134 ± 0.023	
octanal	91	1299	0.00 ± 0	0.00441 ± 0.0013	
nonanal	98	1398	0.00 ± 0	0.00413 ± 0.0011	
(*E*)-2-butenal	95	1046	0.00 ± 0	0.231 ± 0.051	
(*E*)-2-pentenal	93	1147	0.00 ± 0	0.166 ± 0.017	
(*E*)-2-hexenal	98	1230	1.20 ± 0.73	0.168 ± 0.046	4,* 26
(*E*)-2-heptenal	96	1334	0.00 ± 0	0.156 ± 0.033	
(*E*)-2-octenal	96	1434	0.00 ± 0	0.0949 ± 0.021	
(*E*)-2-nonenal	91	1532	0.00474 ± 0.0018	0.00844 ± 0.0013	9*
(*E*)-2-decenal	90	1634	0.00 ± 0	0.00197 ± 0.00085	
2-methyl-(*E*)-2-butenal	94	1113	0.00 ± 0	0.717 ± 0.12	9,* 21, 31*
2-ethyl-(*E*)-2-butenal[f]	94	1166	0.00 ± 0	0.0263 ± 0.0045	
2-methyl-(*E*)-2-pentenal	97	1176	4.76 ± 0.83	0.203 ± 0.080	4,* 9,* 21, 22, 23, 24, 25, 26, 28, 31*
(*E,Z*)-2,4-heptadienal	91	1464	0.0600 ± 0.019	0.0181 ± 0.0045	
(*E,E*)-2,4-heptadienal	95	1490	0.0307 ± 0.0098	0.0137 ± 0.0079	9*
(*E,Z*)-2,4-nonadienal	93	1650	0.00 ± 0	0.0111 ± 0.0023	
(*E,E*)-2,4-nonadienal	94	1686	0.00 ± 0	0.0647 ± 0.018	
(*E,Z*)-2,4-decadienal	94	1746	0.0698 ± 0.027	0.211 ± 0.106	
(*E,E*)-2,4-decadienal	94	1789	0.129 ± 0.053	0.594 ± 0.24	9*
ketones					
2-octanone[f]	95	1297	0.00 ± 0	0.0250 ± 0.0085	
3-octanone	95	1272	0.00 ± 0	0.0165 ± 0.0013	
3-octen-2-one[f]	90	1345	0.00 ± 0	0.0327 ± 0.0060	
3,5-octadien-2-one[f]	93	1516	0.00 ± 0	0.00423 ± 0.00073	
6-undecanone[f]	93	1527	0.00 ± 0	0.00157 ± 0.00029	
β-ionone	98	1910	0.00 ± 0	0.00189 ± 0.00041	
alcohols					
1-pentanol	90	1274	0.00 ± 0	0.484 ± 0.061	
1-hexanol	90	1371	0.00 ± 0	0.184 ± 0.035	
1-octen-3-ol	90	1458	0.00 ± 0	0.0913 ± 0.010	
miscellaneous					
2,5-dimethylfuran[f]	95	958	0.00 ± 0	0.436 ± 0.034	
2-pentylfuran	91	1249	0.104 ± 0.024	0.0862 ± 0.020	

[a] Quality of the GC-MS identification. [b] Retention time index. [c] Standard deviation. [d] Figures correspond to references given in the literature cited section. *, investigations were carried out on leek. Nonmarked records were made on other *Allium* species. [f] Compound was identified by GC-MS data alone.

times the threshold. All of the (*E*)-2-monounsaturated aldehydes—except (*E*)-2-hexenal—increase during the months of storage (**Figure 4**), and only (*E*)-2-hexenal and (*E*)-2-nonenal are found in the fresh leek, whereas the others are formed during storage. After 12 months of storage, the concentration of (*E*)-2-octenal is ~330 times the odor threshold and (*E*)-2-nonenal is present at ~40 times the odor threshold with a maximum of 130 times the threshold at 6 months of storage [0.00034 ppm for (*E*)-2-octenal and 0.00023 ppm for (*E*)-2-nonenal (*17*)]. The concentration of (*E*)-2-hexenal is high in the fresh leeks, which agrees with the results of Ferary and Auger (*4*), who found this compound to be the predominant peak. The decline over time could indicate further conversion to (*E*)-2-hexenol or (*Z*)-2-hexenal, but no traces of these compounds were found in any of the samples.

(*E,E*)-2,4-Decadienal follows the same pattern as hexanal (**Figure 5**). The ratio of produced hexanal to (*E,E*)-2,4-decadienal suggests that the production of these aroma compounds during a frozen storage is due to not only autoxidation but also enzymatic activity, because autoxidation is not specific to one position on the fatty acid. This ratio also indicates that lipoxygenase isolated from leeks is producing hydroperoxides in both the C_{13} and C_9 positions on the fatty acids. LOX is more specific toward the 13-hydroperoxide though, because the concentration of hexanal is ~10 times larger than that of (*E,E*)-2,4-decadienal. (*E,E*)-2,4-Nonadienal is also formed during frozen storage, whereas (*E,E*)-2,4-heptadienal diminishes. (*E,E*)-2,4-Nonadienal and (*E,E*)-2,4-decadienal have been shown to be very potent off-flavors, with threshold values of, respectively, 0.0017 and 0.045 ppb (*17*), which are far below the concentra-

Table 2. Statistical Calculations of Data Displayed in **Figures 2–6**

compound	storage period (months)[a] 0	1	2	4	6	9	12
2-methyl-2-pentenal	B	A	B	C	D	DE	E
3,4- or 2,4-dimethylthiophene	A	ABC	A	AB	BCD	D	CD
dipropyl disulfide	A	A	A	B	B	B	B
propyl (*E*)-propenyl disulfide	A	B	B	C	C	C	C
propyl (*E*)-propenyl trisulfide	C	AB	A	AB	B	C	D
pentanal	E	D	C	A	AC	A	A
hexanal	C	C	C	C	C	B	A
heptanal	C	C	C	C	B	B	A
octanal	C	C	C	C	B	B	A
nonanal	C	B	B	AB	B	AB	A
(*E*)-2-hexenal	A	BC	AB	BC	CD	CD	D
(*E*)-2-heptenal	E	D	BCD	CD	BC	A	AB
(*E*)-2-octenal	D	D	D	C	B	AB	A
(*E*)-2-nonenal	C	C	C	BC	A	AB	BC
(*E*)-2-decenal	C	C	C	C	B	B	A
(*E,E*)-2,4-heptadienal	A	A	A	A	AB	BC	C
(*E,E*)-2,4-nonadienal	D	D	C	C	B	A	A
(*E,E*)-2,4-decadienal	C	C	BC	BC	B	BC	A
LOX activity	AB	A	AB	AB	AB	BC	C
LOX lag phase	AB	B	AB	AB	B	AB	A

[a] Different letters in a row indicate difference on a significance level of 5%.

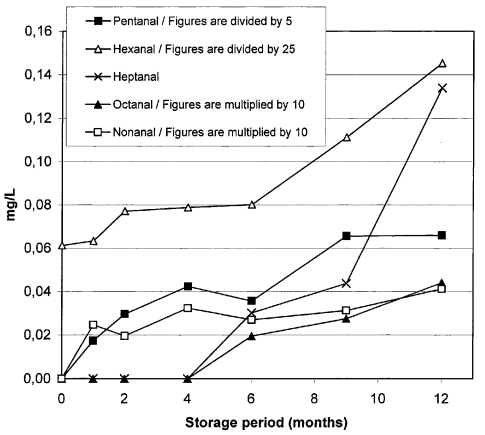

Figure 3. Development of saturated aldehydes in leek slices during frozen storage. See **Table 2** for specification of statistical differences.

tion found in the leek slices after 12 months of frozen storage (**Table 1**). (*E,E*)-2,4-Heptadienal originates from the 12-hydroperoxide (*18*) and is consequently not produced by LOX, which might be the explanation for the different pattern of this aroma compound.

The formation of these aldehydes partly depends on the LOX-catalyzed oxidation of polyunsaturated fatty acids. Lipoxygenase showed a diminishing activity throughout the frozen storage, although ~25% of the original activity was still present after 12 months (**Figure 6**).

LOX activity is measured in a standard assay at optimal conditions of temperature and substrates, which gives no direct indication if the enzyme is actually active in frozen leek, only that it is able to show activity when the slices are crushed prior to the dynamic headspace analysis and oxygen is present in excess. When purged with nitrogen, the atmosphere becomes anaerobic within ~5 min, which means that LOX activity stops. Autoxidation of fatty acids could also produce these compounds (*10*), and both ways will cause accumulation in the tissue.

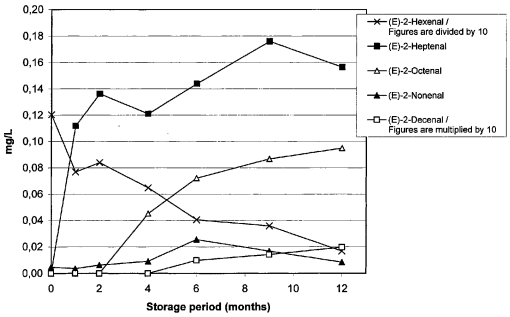

Figure 4. Development of monounsaturated (*E*)-2-aldehydes in leek slices during frozen storage. See **Table 2** for specification of statistical differences.

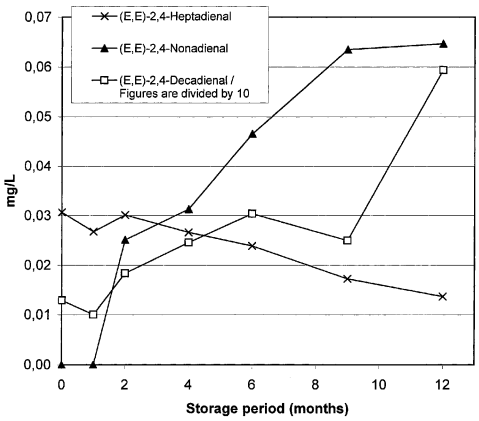

Figure 5. Development of diunsaturated (*E,E*)-2,4-aldehydes in leek slices during frozen storage. See **Table 2** for specification of statistical differences.

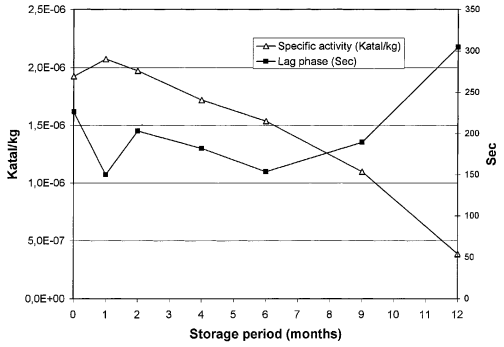

Figure 6. Specific activity (left *y*-axis) and lag phase (right *y*-axis) of lipoxygenase analyzed in leek slices during frozen storage. See **Table 2** for specification of statistical differences.

Autoxidation during the period of tempering and purging in the aroma analysis should be the same at every sampling time during storage, whereas lipoxygenase activity changes; it would be interesting to separate the two processes by inactivating the enzymes before aroma analysis. A number of ketones were also

found to develop during frozen storage, which could originate from autoxidation of polyunsaturated fatty acids (*10*).

Lipoxygenase contains 1 mol of non-heme iron per mole of enzyme, which is essential for catalytic activity (*19*). To catalyze a reaction, LOX must be in the oxidized form (Fe^{3+}), which only ~1% of the native enzyme is (*20*). Oxidation is usually done by its own lipid hydroperoxide product; consequently, a lag phase is present until total activity is reached. **Figure 6** shows the length of the lag phase throughout the frozen storage, which increases. There are several reasons to believe that LOX would be activated in the tissue when the leek slices were stored for a longer period. There is the possibility that more and more lipid hydroperoxides would generate through storage because compartmentation of lipoxygenase and the polyunsaturated fatty acids could be ruined over a freezing period due to loss of cell structure during the growth of ice crystals. Also, more free fatty acids could be available due to lipase activity, and reactions could occur in the phase of liquid water during frozen storage. These results and the decreasing activity indicate a considerable loss of LOX in the tissue during frozen storage.

Table 1 states which compounds have been found by other authors, and it is noticeable that all of the sulfur compounds except methyl pentyl sulfide are in agreement with other investigations performed on *Allium* species. Most of the compounds originating from the LOX pathway and/or the autoxidation route have been found for the first time by this work. This is closely related to the fact that these compounds generate during frozen storage, and all of the studies used for comparison, except ref *9*, have been performed on fresh leeks.

CONCLUSION

The aroma profile of leek slices undergoes a remarkable alteration during 12 months of frozen storage when compared with freshly cut leeks. There is a substantial decrease of the characteristic sulfur compounds, and the concentration of saturated and unsaturated aldehydes increases as an expression of lipoxygenase activity and/or autoxidation of polyunsaturated fatty acids. The catalytic activity of LOX in frozen leek is present throughout a storage term of 12 months, although it is declining and the lag phase is slightly increasing. When leeks are not blanched prior to freezing, the metabolism of fatty acids could be a limitation of the shelf life of the product due to the formation of off-flavor, and there is no doubt that the flavor of frozen leeks stored for 4−6 months or more is quite different from that of fresh leeks.

ABBREVIATIONS USED

LOX, lipoxygenase; FL, concentration in fresh leek; 12M, concentration after 12 months of frozen storage.

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Paper 2

Nielsen G. S.; Poll, L. Impact of water blanching on the retention and formation of aroma compounds in leeks (*Allium ampeloprasum* Var. *Lancelot*).

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Impact of water blanching on the retention and formation of aroma compounds in leeks *(Allium Ampeloprasum Var. Lancelot)*

Ghita Studsgaard Nielsen, Leif Poll

The Royal Veterinary and Agricultural University. Department of Dairy and Food Science, Rolighedsvej 30, 1958 Frederiksberg C. Denmark.

Abstract

The impact of water blanching on the aroma profile of leeks was investigated at 6 blanching times (0, 0.5, 1, 2, 3 and 4 minutes). The aroma profile of fresh cut leeks is dominated by thiosulphinates, which will decompose to sulphides during the blanching procedure. These sulphides increase during blanching times up to 2 minutes, but decrease when blanching time goes beyond that. At the same time a considerable content of sulphur compounds is detected in the blanching water. Lipoxygenase (LOX) generated compounds decrease effectually by rising blanching time, and catalytic activity of LOX was lost with 3 minutes blanching time.

Introduction

Production of frozen vegetables almost always includes blanching both to prevent enzymatic activity, to degas the tissue and to decline the level of spoilage bacteria. Blanching however, also affects the aroma profile of the product (Shamaila *et al.*, 1996; Petersen *et al.*, 1996). This is often evaluated after frozen storage - consequently not only the direct impact of blanching but also alterations during storage are observed. The present study examines the impact of water blanching on aroma compounds and lipoxygenase (EC 1.13.11.12) activity in leek slices.

Experimental

Blanching process

500 g of the white part of leeks was cut into 15 mm slices and immediately after immersed in a pot with lid containing 1 litre of boiling water. Blanching times were 0, 0.5, 1, 2, 3 & 4 minutes and were measured right after immersion. Each time was repeated twice. After blanching leek slices were dripped off for 15 sec and cooled down in crushed ice for 15 minutes prior to analysis. Temperature was measured in the middle of the leek slices and in the water during blanching. Weight alteration after blanching was measured.

Analyses

Each batch of leeks and blanching water was analysed for volatiles (3 replicates), catalytic activity of lipoxygenase (not the water) (2 replicates), dry matter and soluble solids. Volatiles were trapped on Tenax GR using dynamic headspace on blended leek slices or blanching water. Desorption was done thermally by an ATD 400 (Perkin-Elmer). Identification and quantification were performed by GC-MS (HP G1800 A GCD system) and by standard compounds.

Lipoxygenase (LOX) activity was assayed by measuring the consumption of initial dioxygen in an YSI 5100 electrode (Radiometer) at 30°C and pH 6.0 using sodium linoleate as the other substrate.

Results and discussion

The observed changes due to blanching were reflected in the decreasing of some aroma compounds and the increasing of others when compared to raw leeks. This was strongly affected by the length of blanching time and could be explained by the fact that volatile compounds are either formed due to enhanced enzymatic activity at slightly elevated temperatures, heat destructed at higher temperatures, leached to the blanching water or vaporised to the air. Sulphur compounds dominate the aroma profile of fresh cut leeks and other Allium species. Alliinase (EC 4.4.1.4) catalyses the transformation of odourless (+)-*S*-alk(en)yl-L-cysteine sulphoxides into volatile thiosulphinates. These thiosulphinates will be present in the headspace of fresh cut leeks, but as they are thermally unstable (Ferary and Auger, 1996), they will decompose to mono- and polysulphides during the blanching procedure.

Except for a decrease (not statistically significant) when blanching time is 0.5 minutes, dipropyldisulphide enlarged during blanching periods up to 2 minutes (figure 1) probably as an expression of enhanced catalytic activity of alliinase prior to heat induced inactivation. The decrease between 3 and 4 minutes is most likely due to leaching of volatiles to the water and vaporisation. Examples of compounds showing more or less the same behaviour are 2,4-dimethyl thiophene and 1-propanethiol. 2-methyl-2-pentenal (break-down product of thiopropanal-*S*-oxide, the lachrymatory factor) on the other hand was found to diminish during blanching and showed very much the same pattern as the LOX originated compounds.

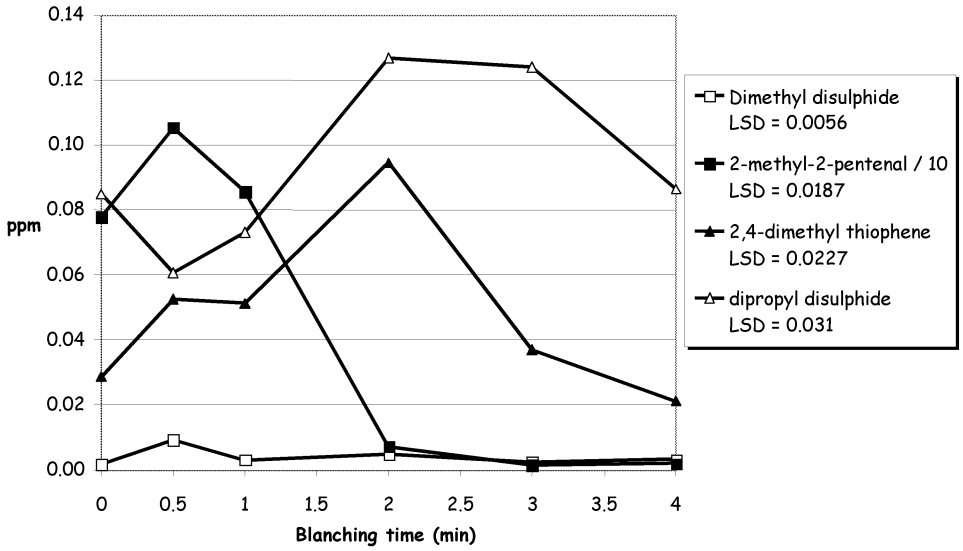

Figure 1. Selected Alliinase generated volatiles in raw and blanched leek slices. Figures for 2-methyl-2-pentenal are divided by 10.

Lipoxygenase generated compounds showed a decreasing behaviour (figure 2). (E)-2-Hexenal and hexanol was completely lost during blanching exceeding 1 minute and hexanal was declining with enhanced blanching time. The catalytic activity of LOX was totally lost after 3 minutes of water blanching, when the temperature in the middle of the leek slices attained approx. 90°C (figure 3), but even 1 minute blanching had a serious impact on the catalytic activity of LOX even though centre temperature only reached 36.4°C. Still this diminished the activity by almost one half compared to the raw leek slices, most likely on account of temperature in outer layers of the leek slices being much higher.

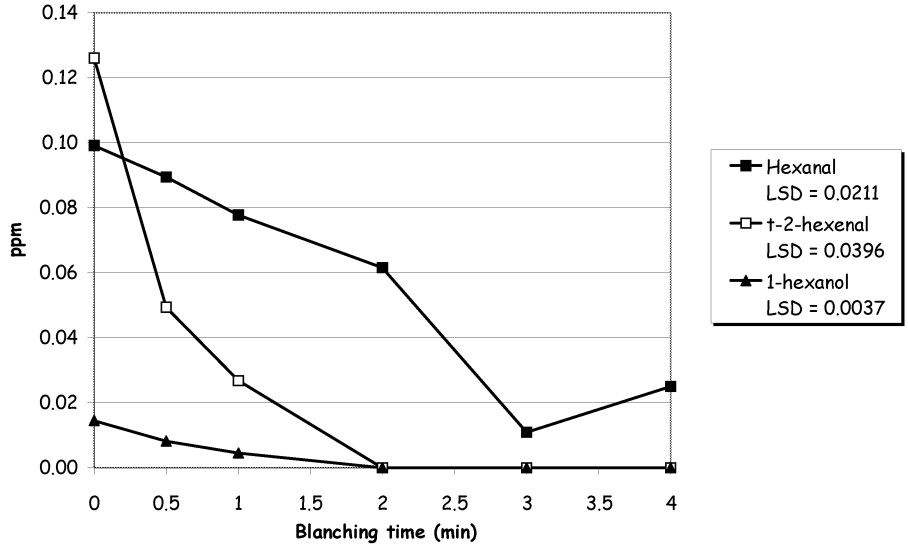

Figure 2. Selected Lipoxygenase generated volatiles in raw and blanched leek slices.

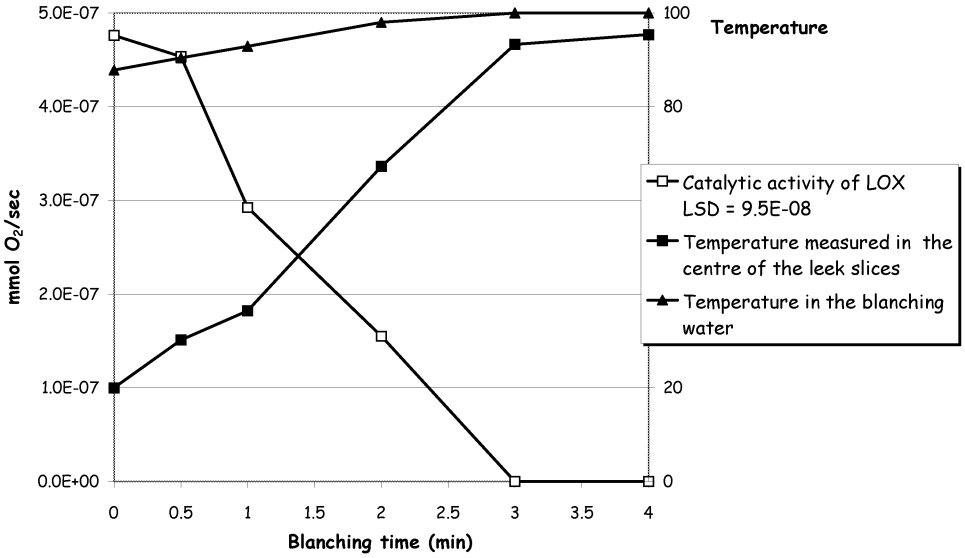

Figure 3. Catalytic activity of Lipoxygenase related to centre temperature of the leek slices.

Analyses on the blanching water revealed a considerable content of sulphur compounds (figure 4), when the blanching time did not exceed 2-3 minutes. LOX generated compounds was found in minor concentrations, (E)-2-hexenal and hexanol was not detected at all. The declining of all volatiles by increasing blanching time was probably due to evaporation to the headspace, as the temperature of the blanching water reached 100°C again 2 minutes after immersing the 20°C leek slices into the boiling water.

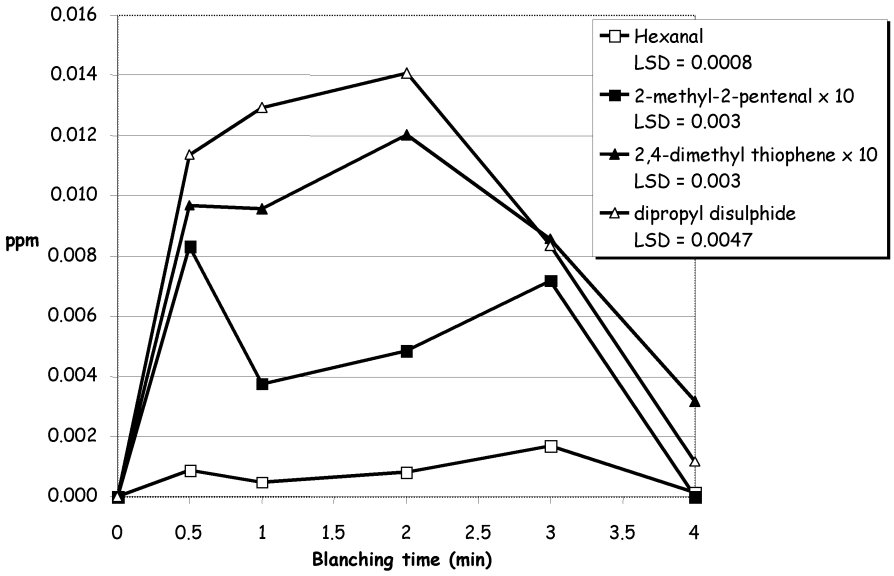

Figure 4. Selected volatiles detected in the blanching water. Figures for 2-methyl-2-pentenal and 2,4-dimethylthiophene are multiplied by 10.

Analysis of soluble solids and dry matter in the blanching water showed increasing contents with enhanced blanching time, whereas contents in leek slices drop to half of the initial value during blanching independent of blanching time (data not shown). An increase of weight of 1-2% was observed after blanching, which was not related to blanching time.

Conclusion

The changes of the aroma profile due to blanching are manifested by the fact that most sulphur compounds increase during shorter blanching times whereas lipoxygenase generated compounds decrease effectively by rising blanching time. Reasons for this observed impact of water blanching on the aroma profile of leeks could be heat induced formation or destruction of volatiles, leaching and vaporisation in combination.

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Paper 3

Nielsen G. S.; Larsen L. M.; Poll L. Formation of aroma compounds during long-term frozen storage of unblanched leek *(Allium ampeloprasum* Var. *Bulga*) as affected by packaging atmosphere and slice thickness.

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Formation of Aroma Compounds during Long-Term Frozen Storage of Unblanched Leek (*Allium ampeloprasum* Var. *Bulga*) as Affected by Packaging Atmosphere and Slice Thickness

GHITA STUDSGAARD NIELSEN,*,† LONE MELCHIOR LARSEN,‡ AND LEIF POLL†

Department of Dairy and Food Science and LMC, Centre of Advanced Food Studies, The Royal Veterinary and Agricultural University, Rolighedsvej 30, 1958 Frederiksberg C, Denmark, and Chemistry Department, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark

Content of aroma compounds and catalytic activity of lipoxygenase (LOX), hydroperoxide lyase (HPL), and alcohol dehydrogenase (ADH) were analyzed in 4- and 15-mm unblanched leek slices packed in atmospheric air (4- and 15-mm) or 100% nitrogen (N) (only 15-mm) seven times during 12 months of frozen storage (12M). Total amount of sulfur compounds was influenced by storage time, slice thickness, and atmosphere (concentration in fresh 4-mm slices = 17.8 mg/L, 4-mm 12M = 3.48 mg/L, fresh 15-mm slices = 2.48 mg/L, 15-mm 12M = 0.418 mg/L and 15-mm N 12M = 1.81 mg/L). The 4-mm slices significantly developed the most aldehydes after 12M (total amount = 9.28 mg/L) compared to 15-mm 12M (6.49 mg/L) and 15-mm N 12M (4.33 mg/L). LOX activity is positively influenced by nitrogen packaging, and HPL activity is influenced by slice thickness, whereas ADH is unaffected by both parameters.

KEYWORDS: Leek; flavor; frozen storage; lipoxygenase; hydroperoxide lyase; alcohol dehydrogenase; nitrogen packaging; slice thickness

INTRODUCTION

The aroma of freshly cut leek and other *Allium* species is dominated by numerous sulfur-containing volatile compounds originating from the alliinase- (EC 4.4.1.4) catalyzed decomposition of the odorless nonvolatile precursors *S*-alk(en)ylcysteine sulfoxides (*1*, *2*).

The products, sulfenic acids, are highly reactive (*3*) and will quickly combine to form thiosulfinates. Thiosulfinates are responsible for the odor of freshly cut leeks (*3*, *4*), but as they are relatively unstable (*1*, *5*), they will rearrange to form polysulfides and thiosulfonates. As thiosulfonates are transformed to the corresponding monosulfides, the final products of the reaction will be a combination of mono- and polysulfides with all of the possible combinations of the alk(en)yl radicals. Prior investigations (*6*) have shown that these sulfur compounds decline during frozen storage of leek slices.

The lipoxygenase pathway also contributes to the aroma formation in fresh and especially in stored leeks. Lipoxygenase (EC 1.13.11.12) (LOX) catalyzes the formation of hydroperoxy derivatives of polyunsaturated fatty acids with a *cis*,*cis*-pentadiene moiety (*7*, *8*) under the consumption of dioxygen. Volatile aldehydes are produced by the action of hydroperoxide lyase (HPL) upon the formed hydroperoxides (*7*, *9*). All of these aldehydes, both saturated and nonsaturated, can be further metabolized by alcohol dehydrogenase (EC 1.1.1.1) (ADH) to the corresponding alcohols (*9*).

This formation of mainly aldehydes and alcohols may contribute to the aroma of fresh leeks but will probably appear as off-flavors when present in larger amounts (*6*, *10*). This is not very pronounced in the fresh leek because of the pungency of thiosulfinates and thiopropanal-*S*-oxide (*5*). However, when leek slices are stored frozen for a longer period, the production of aldehydes will have an influence on the aroma profile as well, both because of the increasing amount of these compounds and because of the diminishing content of sulfur compounds (*6*, *10*). These compounds derived from fatty acids can also be generated by autoxidation during the storage period (*11*), and both pathways will result in accumulation of the products in the tissue.

Previous investigations of unblanched frozen leek slices (*6*) have shown that the storage period has a great influence on the aroma compounds in the leek slices and that the concentration of saturated and unsaturated aldehydes increases effectively during the storage. This paper deals with the impact of changing the headspace in the package of the frozen leek slices from atmospheric air to 100% nitrogen, which to our knowledge has not been investigated before. This could prevent oxidation during the frozen storage, which will result in less accumulation of off flavor in the leek slices. Also, the influence of slice thickness

* To whom correspondence should be addressed. Tel.: +45-35283435. Fax: +45-35283265. E-mail gni@kvl.dk.
† Centre of Advanced Food Studies.
‡ Chemistry Department.

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on the development of aroma compounds during frozen storage is investigated.

MATERIALS AND METHODS

Plant Material. Leeks (*Allium ampeloprasum* var. *Bulga*) were harvested fully matured at Funen, Denmark in October 2000 and used for the experiment immediately after.

Chemicals. 1-Propanethiol, dimethyl disulfide, methyl propyl disulfide, dipropyl disulfide, dimethyl trisulfide, 2,5-dimethylthiophene, propanal, butanal, pentanal, hexanal, heptanal, octanal, nonanal, decanal, (*E*)-2-butenal, (*E*)-2-pentenal, (*E*)-2-hexenal, (*E*)-2-heptenal, (*E*)-2-octenal, 2-methyl-(*E*)-2-butenal, 2-methyl-(*E*)-2-pentenal, (*E*,*E*)-2,4-heptadienal, (*E*,*E*)-2,4-nonadienal, 3-octanone, 1-pentanol, 1-hexanol, 1-octen-3-ol, and 2-pentyl furan were bought commercially from Sigma-Aldrich, Copenhagen, Denmark. (*E*,*Z*)-2,4-Heptadienal was occurring as an impurity in (*E*,*E*)-2,4-heptadienal. All other chemicals, except 9-(S)-hydroperoxy-(10E,12*Z*)-octadecadienoic acid, were of analytical grade and were bought commercially from Sigma-Aldrich.

Synthesis of 9-(*S*)-Hydroperoxy-(10*E*,12*Z*)-octadecadienoic Acid (9-HPODE). Sodium linoleate (1.25 mL, 10 mM), 4.75 mL of air saturated 50 mM potassium phosphate buffer (pH 7.0), and 1.50 mL of potato tuber lipoxygenase solution were mixed then agitated and purged with oxygen for 30 min. The solution was checked for absorbance at 234 nm to determine the content of 9-HPODE. According to Galliard and Phillips (*12*), potato tuber LOX almost exclusively converts linoleic acid into 9-HPODE.

Preparation of Potato Tuber Lipoxygenase Solution for Synthesis. Grated potato tubers (50 g) and 50 mL of tap water were homogenized for 30 s in a Waring commercial blender and filtrated through a paper filter. A 2-mL aliquot of the liquid was applied to a DEAD-Cellulose C545 column (anion exchanger) followed by 6.0 mL of 50 mM potassium phosphate buffer (pH 7.0). Two fractions of 4.0 mL were collected; both were checked for activity, and the second one was used as the lipoxygenase solution.

Sample Preparation. The white part of the leek stem was cut into 4-mm slices or 15-mm slices and frozen immediately after in a blast freezer at −20 °C for 10 min, so that the center temperature reached −20 °C. The slices were packed in airtight glass jars with atmospheric air or 100% nitrogen (only 15-mm slices) as headspace and kept at −20 °C in the dark until analysis. Fresh Pax oxygen absorbers (Type R 50 CC) from Multisorb Technologies, Inc. Buffalo, NY were added to the glasses with 100% nitrogen.

Experimental Design. Two individual samples of 4-mm slices and 15-mm slices were analyzed at harvest time, and subsequently, two individual samples of 4,- 15-, and 15-mm N were analyzed after 1, 2, 4, 6, 9, and 12 months of storage. Each sample was analyzed for composition of atmosphere in the glass jars, catalytic activity of lipoxygenase, hydroperoxide lyase, and alcohol dehydrogenase, and content of aroma compounds in three replicates.

Gas Analysis. The O_2, CO_2, and N_2 concentrations in the glass jars were determined by a Gaspace 2 analyzer from Systech Instruments Ltd, Oxon, UK.

Preparation of Enzyme Extract. Enzyme extract was made by mixing 100 g of frozen leek slices with 100 mL of potassium phosphate buffer (50 mM, pH 7.0 added 0.1% Triton X-100) for 120 s in a Waring commercial blender. The slurry was kept on ice with agitation for 30 min (100 rpm), afterward filtered through a paper filter until 35.0 mL was collected, and kept on ice until time of analysis.

Lipoxygenase Assay. LOX was assayed in a continuous assay by measuring consumption of initial dioxygen by an YSI 5100 dissolved oxygen meter (YSI Inc., Yellow Springs, OH) at 30 °C, using linoleic acid as the other substrate. Calibration was done at 30 °C by air-saturated phosphate buffer (0.2 M, pH 6.0) (21% dissolved dioxygen) followed by addition of sodium dithionite (0% dissolved oxygen). To determine lipoxygenase activity, 27.2 mL of air-saturated tempered phosphate buffer (0.2 M, pH = 6.0) and 3.9 mL of enzyme extract was mixed in a 33-mL conical flask, and the measuring was started. After 30 s, the reaction was initiated by adding 1.9 mL of sodium linoleate solution (10 mM). Dioxygen was monitored over 20 min, measuring dioxygen content every second.

Blank samples were run on buffer added substrate or enzyme extract.

Calculation of LOX Activity. LOX activity was calculated as katals per kg of protein, and katals were defined as moles of O_2 per second. This was done under the assumption that air-saturated phosphate buffer (0.2 M, pH 6.0) at 30 °C with a salt strength of 12.40 g/L contains 7.11 mg O_2/L (*13*). To determine the activity the dioxygen level (mmol O_2) was plotted against time and the steepest slope (mmol O_2/s) on the curve was found using continuous linear regression over 20 s at a time.

Hydroperoxide Lyase Assay. HPL was assayed in a continuous coupled assay modified after (*14*). ADH converts the products of HPL's reaction on hydroperoxides, aldehydes, under the oxidation of NADH, which can be monitored spectrophotometrically at 340 nm.

Phosphate buffer (1.8 mL, 0.2 M, pH = 6.0), 300 μL of 2.2 mM NADH, 200 μL of ADH (150 units in 100 μL) and 300 μL of enzyme extract (filtrated through 1.2-μm and 0.45-μm filters from Orange Scientific, Braine-l'Alleud, Belgium) were transferred to a thermostatic (30 °C) 1-cm cuvette with a magnetic stirrer, and the measuring was started. After 30 s, 400 μL of 9-HPODE was added. The reaction was monitored over 600 s.

Blank samples were run on buffer, NADH, and ADH added substrate or enzyme extract.

Calculation of HPL Activity. HPL activity was calculated as katals per kg of protein and katals were defined as moles consumed of hydroperoxide/s. To determine the activity, the absorbance was plotted against time, and the steepest slope (absorbance/s) on the curve was found using continuous linear regression over 5 s at a time. Absorbance was converted to moles of hydroperoxide by the assumption that each mole of hydroperoxide metabolized produced one mole of aldehyde. Aldehydes are converted by alcohol dehydrogenase by the consumption of NADH 1:1. ϵ of NADH at 340 nm is 6220 L/mol · cm (*14*).

Alcohol Dehydrogenase Assay. ADH was assayed in a continuous assay by following the oxidation of NADH spectrophotometrically at 340 nm when adding hexanal.

Borate buffer (1.4 mL, 0.05 M, pH = 9.0), 300 μL of 2.2 mM NADH and 1000 μL of enzyme extract (filtrated through 1.2-μm and 0.45-μm filters from Orange Scientific,′) were transferred to a thermostatic (30 °C) 1-cm cuvette with a magnetic stirrer, and the measuring was started. After 30 s, 300 μL of hexanal (100 ppm) was added. The reaction was monitored over 600 s.

Blank samples were run on buffer and NADH added substrate or enzyme extract.

Calculation of ADH Activity. Alcohol dehydrogenase activity was calculated as katals per kg of protein, and katals were defined as moles of hexanal/s. Moles of hexanal were calculated on the basis of NADH under the assumption that hexanal and NADH were metabolized 1:1 and by the conditions described under calculation of HPL activity.

Determination of Protein Content. Protein content in the enzyme extraction was determined by Coomassie brilliant blue, according to the method described in US/EG Bulletin 1069 from Bio-Rad Life Science Group, Hercules, CA, using lyophilized bovine serum albumin as the standard protein.

Dynamic Headspace Analysis. Aroma compounds were isolated by dynamic headspace with nitrogen (purity = 99.8%) as purge gas. Fresh or frozen leek slices (100 g) were crushed with 150 mL of tap water and 4 mL of internal standard (50 ppm of 4-methyl-1-pentanol in tap water) for 120 s in a Waring commercial blender. After the mixture had been transferred to a 1-L glass flask by adding another 150 mL of tap water, it was left for 10 min at 30 °C with agitation (200 rpm) to equilibrate the temperature before purging. Aroma compounds were trapped on 250 mg of Tenax GR (mesh size = 60/80, Buchem bv, Apeldoorn, The Nederlands) for 45 min with a nitrogen flow of 75 mL/min and agitation (200 rpm).

Desorption of aroma compounds was done thermally by an ATD 400 automatic thermal desorption system (Perkin-Elmer, Bucks, England). Desorption temperature of the trap to the cold trap (contains 30 mg Tenax GR, 5 °C) was 250 °C for 15 min with a helium flow of 60 mL/min. Desorption temperature of the cold trap was 300 °C for 4 min, with a helium flow of 31 mL/min and an outlet split ratio of 1:30. Separation was performed by a GC-MS (HP G1800 A GCD system) with the following conditions: column, DB Wax from J&W Scientific, CA (30-m × 0.25-mm i.d. × 0.25-μm film thickness); carrier gas,

Table 1. Atmosphere Composition in % in the Glass Jars during Storage of Frozen Leek Slices

storage period (months)	4 mm			15 mm			15 mm N		
	O_2	CO_2	N_2	O_2	CO_2	N_2	O_2	CO_2	N_2
1	18.5	3.2	78.3	18.7	3.5	77.8	0.5	2.5	97.0
2	19.1	2.2	78.7	18.6	3.8	77.6	0.9	2.0	97.1
4	18.1	1.8	80.1	17.4	3.5	79.1	0.9	1.8	97.3
6	17.7	1.8	80.5	15.7	4.9	79.4	0.1	2.2	97.7
9	17.1	1.8	81.1	14.8	4.5	80.7	0.4	1.7	97.9
12	16.4	2.7	80.9	13.6	6.4	80.0	0.1	2.0	97.9

helium; start flow, 1 mL/min (constant); split ratio, none; column pressure (constant), 48 kPa; oven program, 45 °C for 10 min, 6 °C/min to 240 °C, constant at 240 °C for 30 min. The mass selective detector was operated in the electron ionization mode (ionization energy, 70 eV), and the *m/z* (mass/charge) ratio ranged between 10 and 425. Identification was done by probability-based matching with mass spectra in the G1035A Wiley library (Hewlett-Packard). Identity was confirmed by checking with mass spectra and retention indices obtained in the laboratory from reference compounds for the following compounds: 1-propanethiol, dimethyl disulfide, methyl propyl disulfide, dipropyl disulfide, dimethyl trisulfide, 2,5-dimethylthiophene, propanal, butanal, pentanal, hexanal, heptanal, octanal, nonanal, decanal, (*E*)-2-butenal, (*E*)-2-pentenal, (*E*)-2-hexenal, (*E*)-2-heptenal, (*E*)-2-octenal, 2-methyl-(*E*)-2-butenal, 2-methyl-(*E*)-2-pentenal, (*E*,*Z*)-2,4-heptadienal, (*E*,*E*)-2,4-heptadienal, (*E*,*E*)-2,4-nonadienal, 3-octanone, 1-pentanol, 1-hexanol, 1-octen-3-ol, and 2-pentyl furan.

Quantification. A 10-μL aliquot of each of the above listed reference compounds was dissolved in 20 mL of 96% ethanol and diluted with tap water into four appropriate concentrations depending on the concentration of the compounds in the leeks. Sucrose (1.3%), which equals the sugar content of leeks, was added to the final solutions. Dynamic headspace analysis performed on 400 mL of the solutions added 4 mL of internal standard with the same flow, time, and temperature conditions as applied to the leek samples. Each reference compound was analyzed in triplicate in all four concentrations. The obtained peak area divided by the peak area of the internal standard was used to calculate the concentration of the same compound in the leeks from the peak area of the compound divided by the peak area of the internal standard. Methyl 2-propenyl disulfide, methyl propenyl disulfide, and ethyl 1-methylethyl disulfide were quantified after the obtained peak area of methyl propyl disulfide, propyl 2-propenyl disulfide; propyl propenyl disulfide after dipropyl disulfide, methyl propyl trisulfide, diisopropyl trisulfide; propyl propenyl trisulfide after dimethyl trisulfide; 3,4- or 2,4-dimethyl thiophene after 2,5-dimethyl thiophene; 2-ethyl-(*E*)-2-butenal after 2-methyl-(*E*)-2-pentenal, 3-octen-2-one; 3,5-octadiene-2-one after 3-octanone; and 2,5-dimethyl furan after 2-pentyl furan

The retention time indices (RTI) of the volatile compounds were calculated with a mixture of hydrocarbons (C_9−C_{26}) as references, according to the method of (*15*).

Statistical Analysis. Analysis of variance was carried out on LOX, HPL, and ADH and on each of the aroma compounds using the one-way and the factorial ANOVA procedure in the Analyst part of SAS, version 8.2, SAS Institute Inc., Cary, NC. The effect of the storage period and the processing method was tested. Sample means were compared by Duncan's multiple range test. A significance level of 5% was applied.

RESULTS AND DISCUSSION

The actual composition of the atmosphere in the glass jars at each sampling time is displayed in **Table 1**. The nitrogen packaging keeps very constant at 97−98% N_2 and below 1% of O_2. The 4- and 15-mm slices both modify the atmosphere in the jars packed with atmospheric air. The 15-mm slices are most effective in changing the atmosphere, which ends at 6.4% CO_2 and 13.6% O_2, suggesting that respiration occurs in the leek slices during the frozen storage and that the degree of intact tissue per slice has an impact on this activity.

A complete overview over the aroma compounds detected in leek slices, fresh and after 12 months of frozen storage, is given in **Table 2** for all three treatments (4-, 15-, and 15-mm N slices). Of the detected sulfur compounds, the aroma is dominated by dipropyl disulfide, especially in the fresh leek slices, which is consistent with what Keusgen et al. (*16*) found in various *Allium* hybrids. There is a noticeable effect of the slicing thickness as the 4-mm fresh cut slices produces significantly more of all of the sulfur compounds compared to the other treatments shown in **Table 2**, the exceptions being equal amounts of dimethyl disulfide and 2,5-dimethyl thiophene produced in 4- and 15-mm fresh slices and diisopropyl trisulfide produced in 4- and 15-mm N 12M slices. The sulfur volatiles are not produced in the intact cells because of compartmentation of alliinase in the vacuole and the cysteine sulfoxides in the cytoplasm (*2*, *17*), but during the cleaning and slicing process, the formation of sulfur compounds starts. The 4-mm slices have a larger percent of disrupted cells per weight unit than the 15-mm slices have, which can explain this observed difference. During the headspace collection of volatiles, though, formation of sulfur compounds might be possible because the slices are totally crushed during the sample preparation procedure. Purging with nitrogen quickly creates anaerobic conditions in the leek slurry, but alliinase is not dependent on oxygen being present as lipoxygenase is.

This observed effect of the slice thickness is not only valid for the fresh leek slices, as there are also significant differences between slice thicknesses when the leek slices have been stored frozen for 12 months. For many sulfur compounds, such as dipropyl disulfide, 4-mm slices have a tendency to have the largest concentration throughout the storage period. The same pattern is found for methyl propyl disulfide, ethyl 1-methylethyl disulfide, propyl propenyl disulfide, propyl 2-propenyl disulfide, and diisopropyl trisulfide. For 4-mm slices all of the sulfur compounds decreased effectually during frozen storage, which is in agreement with what is already found for 15-mm slices (*6*). In the present study, 15-mm slices also show significant changes for most of the sulfur compounds during 12 months of frozen storage (e.g., dimethyl disulfide (**Figure 1**) decreases significantly). **Table 3** presents the statistical differences of data displayed in **Figures 1**−**5**.

In contrast to our previous investigations (*6*), this experiment shows that all of the sulfur compounds are still present after 12 months of frozen storage in both slice thicknesses and in the nitrogen packaging. While most of the compounds follow the pattern of **Figure 1**, 2,5-dimethyl thiophene is present at a constant concentration from 2 to 9 months of storage, except for a high value found after 1 month of storage, and is only decreased during the last 3 months of storage.

When comparing the total amount of sulfur compounds, the effect of frozen storage stands out clearly. The 4-mm slices alter from 17763 μg/L in the fresh leeks to 3475 μg/L after 12 months of frozen storage, and the 15-mm fresh leek slices contain 2480 μg/L, whereas leek slices frozen for 12 months contain 418 μg/L (**Table 2**), which in both cases are significant changes.

Nitrogen packaging has a great influence on the keeping of the sulfur compounds (**Table 2**). Half of the sulfur compounds are found in equal concentrations after 12 months of frozen storage under nitrogen compared to the fresh 15-mm slices, and two even in higher concentrations. Diisopropyl trisulfide is present in 425 μg/L, which is an unexpected high part of the total amount (1809 μg/L) of sulfur compounds that were

Table 2. Aroma Compounds Found in Fresh Leek and in Leeks Frozen for 12 Months (12 M)[a]

compound	RTI[b]	4-mm fresh leek	4-mm 12 M	15-mm fresh leek	15-mm 12 M	15-mm N[c] 12 M
			Sulfur Compounds			
1-propanethiol	857	260 ± 197	36.2 ± 6.88	54.7 ± 38.5	19.8 ± 7.15	55.3 ± 24.4
dimethyl disulfide	1086	170 ± 55.4	11.0 ± 4.50	137 ± 130	2.21 ± 1.77	5.00 ± 3.32
methyl propyl disulfide	1242	1314 ± 586	318 ± 104	257 ± 156	27.4 ± 19.5	129 ± 33.9
methyl 2-propenyl disulfide[e]	1296	0.635 ± 0.231	0.0826 ± 0.0237	0.208 ± 0.0861	0.0131 ± 0.0141	0.0418 ± 0.00869
methyl propenyl disulfide[e,f]	1263	312 ± 110	4.69 ± 1.19	85.3 ± 26.8	2.16 ± 1.45	7.58 ± 1.98
methyl propenyl disulfide[e,f]	1292	1225 ± 443	19.1 ± 5.87	250 ± 72.8	6.94 ± 4.20	21.5 ± 5.98
ethyl 1-methylethyl disulfide[e]	1319	7.87 ± 3.21	4.35 ± 1.05	0.993 ± 0.349	0.595 ± 0.247	2.38 ± 0.643
dipropyl disulfide	1387	11076 ± 4249	2630 ± 590	1250 ± 320	208 ± 67.3	966 ± 260
propyl 2-propenyl disulfide[e]	1436	1.64 ± 0.687	0.486 ± 0.138	0.124 ± 0.0521	0.0137 ± 0.0151	0.257 ± 0.415
propyl propenyl disulfide[e,f]	1422	115 ± 62.8	14.8 ± 3.47	20.3 ± 8.65	4.42 ± 1.60	15.5 ± 4.69
propyl propenyl disulfide[e,f]	1438	1520 ± 720	94.2 ± 22.7	153 ± 89.0	37.7 ± 21.8	58.3 ± 22.7
dimethyl trisulfide	1376	231 ± 94.2	1.70 ± 0.604	40.9 ± 10.5	0.573 ± 0.806	4.76 ± 1.26
methyl propyl trisulfide[e]	1531	361 ± 178	21.7 ± 6.17	44.3 ± 19.2	5.00 ± 4.25	30.6 ± 11.1
diisopropyl trisulfide[d]	1656	549 ± 239	255 ± 55.2	84.3 ± 56.8	50.2 ± 20.8	425 ± 75.9
propyl propenyl trisulfide[e,f]	1781	20.8 ± 5.06	0.780 ± 0.139	2.48 ± 1.01	0.836 ± 0.252	2.97 ± 0.686
propyl propenyl trisulfide[e,f]	1770	24.8 ± 4.94	1.19 ± 0.238	3.32 ± 1.53	0.886 ± 0.291	3.21 ± 0.709
2,5-dimethyl thiophene	1190	18.9 ± 8.75	1.91 ± 0.382	14.0 ± 5.26	3.00 ± 1.49	6.42 ± 1.22
3,4- or 2,4-dimethyl thiophene[e]	1253	557 ± 484	59.9 ± 11.1	82.7 ± 48.9	47.7 ± 10.5	76.2 ± 15.9
total of sulfur compounds		17763 ± 7340	3475 ± 769	2480 ± 583	418 ± 128	1809 ± 444
			Aldehydes			
propanal	762	8118 ± 1980	1697 ± 630	3683 ± 720	2811 ± 1273	4348 ± 635
butanal	867	0.00 ± 0	25.5 ± 6.19	0.00 ± 0	41.0 ± 11.8	20.1 ± 5.81
pentanal	984	225 ± 104	1426 ± 337	144 ± 15.7	770 ± 41.0	351 ± 759
hexanal	1111	1349 ± 140	4256 ± 1099	1191 ± 397	3178 ± 195	1191 ± 279
heptanal	1197	0.00 ± 0	115 ± 26.4	0.00 ± 0	85.0 ± 3.73	39.5 ± 4.70
octanal	1299	0.00 ± 0	15.9 ± 3.50	0.00 ± 0	8.03 ± 1.23	0.154 ± 0.128
nonanal	1398	0.561 ± 0.364	4.32 ± 3.30	0.0722 ± 0.0179	4.96 ± 0.597	2.71 ± 0.619
decanal	1510	0.00 ± 0	1.14 ± 0.622	0.00 ± 0	0.881 ± 0.393	0.536 ± 0.273
(*E*)–2-butenal	1046	0.00 ± 0	781 ± 237	0.00 ± 0	415 ± 153	264 ± 75.5
(*E*)–2-pentenal	1147	0.00 ± 0	149 ± 39.6	0.00 ± 0	111 ± 26.5	178 ± 12.8
(*E*)–2-hexenal	1230	1275 ± 145	22.3 ± 12.2	1072 ± 881	39.1 ± 23.7	82.0 ± 17.1
(*E*)–2-heptenal	1334	219 ± 34.1	85.5 ± 16.6	122 ± 35.1	81.4 ± 4.47	85.4 ± 7.67
(*E*)–2-octenal	1434	0.783 ± 0.511	0.435 ± 0.394	0.838 ± 1.10	49.1 ± 3.70	18.2 ± 11.6
2-methyl-(*E*)–2-butenal	1113	0.00 ± 0	2349 ± 596	0.00 ± 0	1660 ± 258	2013 ± 283
2-ethyl-(*E*)–2-butenal[e]	1166	0.00 ± 0	17.2 ± 11.5	0.00 ± 0	24.5 ± 8.92	15.5 ± 24.1
2-methyl-(*E*)–2-pentenal	1176	7747 ± 2280	91.9 ± 22.1	5061 ± 954	152 ± 100	506 ± 71.9
(*E,Z*)–2,4-heptadienal	1464	358 ± 38.9	6.63 ± 5.31	141 ± 77.7	10.9 ± 4.50	40.5 ± 8.66
(*E,E*)–2,4-heptadienal	1490	175 ± 57.2	13.4 ± 11.0	45.2 ± 20.9	9.84 ± 1.97	30.1 ± 2.04
(*E,E*)–2,4-nonadienal	1686	0.00 ± 0	12.9 ± 6.08	0.00 ± 0	3.85 ± 6.67	2.20 ± 2.36
total of aldehydes[d]		3602 ± 334	9281 ± 2310	2717 ± 1380	6489 ± 358	4333 ± 553
			Ketones			
3-octanone	1272	0.00 ± 0	11.0 ± 3.30	0.00 ± 0	11.9 ± 2.31	1.38 ± 1.08
3-octen-2-one[e]	1345	0.00 ± 0	7.62 ± 4.20	0.00 ± 0	5.30 ± 0.362	0.487 ± 0.511
3,5-octadiene-2-one[e]	1516	0.00 ± 0	7.83 ± 2.29	0.00 ± 0	4.41 ± 1.43	1.34 ± 0.425
total of ketones		0.00 ± 0	26.5 ± 8.70	0.00 ± 0	21.6 ± 2.35	3.20 ± 1.20
			Alcohols			
1-pentanol	1274	0.00 ± 0	356 ± 87.0	0.00 ± 0	299 ± 40.9	207 ± 22.3
1-hexanol	1371	0.00 ± 0	56.1 ± 35.8	0.00 ± 0	172 ± 80.1	17.4 ± 8.38
1-octen-3-ol	1458	0.00 ± 0	57.1 ± 14.1	0.00 ± 0	46.3 ± 2.60	10.9 ± 7.11
total of alcohols		0.00 ± 0	469 ± 106	0.00 ± 0	517 ± 112	235 ± 29.7
			Furans			
2,5-dimethyl furan[e]	958	0.00 ± 0	4.93 ± 1.30	0.00 ± 0	6.37 ± 2.20	0.261 ± 0.304
2-pentyl furan	1249	55.5 ± 23.3	17.0 ± 3.73	36.3 ± 11.3	17.1 ± 4.04	29.7 ± 6.50
total of furans		55.5 ± 23.3	22.0 ± 4.95	36.3 ± 11.3	23.5 ± 4.87	29.9 ± 6.43

[a] All values except RTI are in μg/L ± standard deviation. [b] Retention time index. [c] N = packed in nitrogen. [d] Except for propanal and 2-methyl-2-pentenal. [e] Compound was identified by GC-MS alone and quantified by peak area of another compound, see Materials and Methods for details. [f] (*Z*) and (*E*) isomers.

detected in the leek slices packed under nitrogen. Most of the sulfur compounds are found in significantly higher concentrations in the nitrogen packed leek slices compared to the 12 months frozen storage of 15-mm slices. Nitrogen packaging prevents the respiratory activity that is viable at −20 °C (**Table 1**), and possibly at the same time the metabolic degradation of sulfur compounds is more or less prevented, resulting in the positive aroma keeping of the sulfur compounds.

Aldehydes show a different development during storage, 9 out of 19 are not found at all in the fresh leek slices, regardless of slice thickness; this applies to butanal, heptanal, octanal, decanal, (*E*)-2-butenal, (*E*)-2-pentenal, 2-methyl-(*E*)-2-butenal, 2-ethyl-(*E*)-2-butenal, and (*E,E*)-nonadienal, and most of the others are present only in very low concentrations in the fresh tissue (**Table 2**). The exceptions are propanal, (*E*)-2-hexenal, (*E*)-2-heptenal, 2-methyl-(*E*)-2-pentenal, and both 2,4-heptadienals, which are all exhibiting the same pattern as dimethyl disulfide (**Figure 1**). (*E*)-2-Hexenal is found by others (*3*) to be present in large amounts in fresh leek and is shown to follow the typical pattern of sulfur compounds (*6*). Propanal and 2-methyl-(*E*)-2-pentenal are both breakdown products from thiopropanal-*S*-oxide (the lachrymatory factor) (*18*), which is not detectable with our GC-method (*6*, *19*). Of the detected aldehydes in the fresh leek slices, there are significant differ-

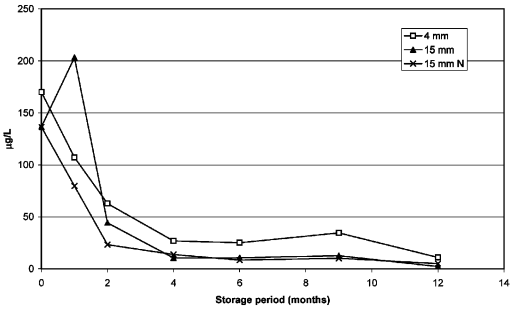

Figure 1. Development of dimethyl disulfide in leek slices during frozen storage. See **Table 3** for specification of statistical differences within one treatment.

Table 3. Statistical Calculations of Data Displayed in **Figures 1–5**

processing method	storage period (months)[a] 0	1	2	4	6	9	12
			Figure 1 Dimethyl Disulfide				
4-mm	A	B	C	D	D	CD	D
15-mm	B	A	C	C	C	C	C
15-mm N	A	AB	BC	C	C	C	C
			Figure 2 Hexanal				
4-mm	E	D	B	A	C		
15-mm	D	CD	CD	C	B	A	B
15-mm N	B	B	B	B	B	A	B
			Figure 3 LOX Activity				
4-mm	A	B	C	C	C	C	C
15-mm	A	B	E	C	DE	CD	CDE
15-mm N	B	C	DE	E	CD	A	C
			Figure 4 HPL Activity				
4-mm	A	A	A	B	B	B	A
15-mm	AB	A	BC	C	C	C	AB
15-mm N	A	A	A	BC	AB	C	A
			Figure 5 ADH Activity				
4-mm	A	AB	A	BCD	CD	BC	D
15-mm	A	A	AB	ABC	BCD	CD	D
15-mm N	A	A	A	B	B	B	B

[a] Different letters in a row indicates difference on a significance level of 5%. Letters cannot be compared among rows.

ences in concentration between the two thicknesses for 6 out of the 10 detected aldehydes, namely propanal, nonanal, (*E*)-2-heptenal, 2-methyl-(*E*)-2-pentenal, and both 2,4-heptadienals, where 4-mm slices generate the largest concentrations.

During frozen storage, slice thickness has a strong impact on development of off flavor, as many of the aldehydes are found in the highest concentration in 4-mm slices (**Table 2**). This is found after 12 months of frozen storage, but applies to the whole storage period. **Figure 2** shows the development of hexanal, which resembles those of pentanal, octanal, (*E*)-2-butenal, (*E*)-2-pentenal, (*E*)-2-heptenal, and (*E*,*E*)-2,4-nonadienal. Compartmentation of LOX in the cytosol and its substrate (fatty acids) in the cell membranes makes the basis for enzymatic activity of LOX and production of aldehydes during frozen storage more favorable in the 4-mm slices because of a larger degree of disrupted cells per weight unit that in the 15-mm slices.

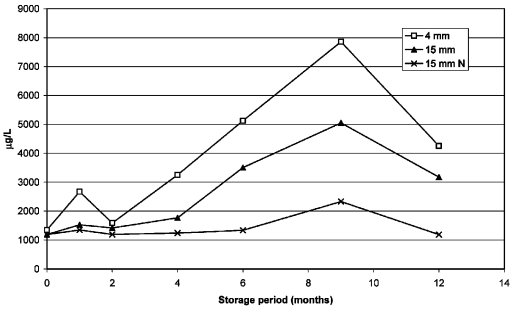

Figure 2. Development of hexanal in leek slices during frozen storage. See **Table 3** for specification of statistical differences within one treatment.

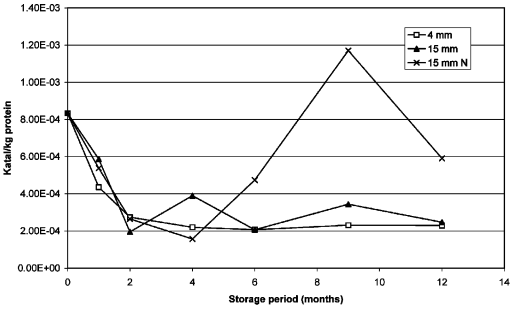

Figure 3. Specific activity of LOX analyzed in leek slices during frozen storage. See **Table 3** for specification of statistical differences within one treatment.

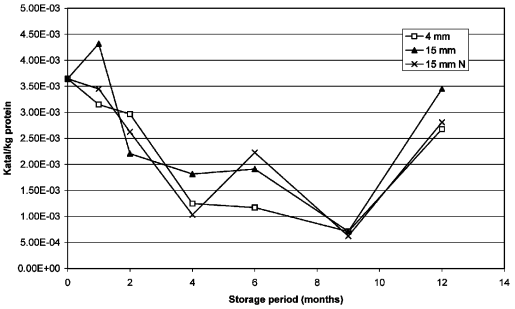

Figure 4. Specific activity of HPL analyzed in leek slices during frozen storage. See **Table 3** for specification of statistical differences within one504 treatment.

Nitrogen packaging is to some extent preventing formation of off-flavor in the leek slices during frozen storage. After 12 months of frozen storage under nitrogen, 11 out of 19 aldehyde compounds found are in the same level as the fresh leek slices or in a lower concentration than the 15-mm slices stored for 12 months. Hexanal (**Figure 2**) is kept almost constant during storage, when nitrogen is present in the glass jar, whereas it develops effectually in atmospheric air headspace. This is also found for butanal, pentanal, heptanal, octanal, (*E*,*E*)-2,4-nonadienal, and hexanol. As the sulfur compounds at the same time are kept at almost the same level as for the fresh leek slices, there is no doubt that the nitrogen packaging is maintaining the aroma profile of the fresh leek much better than the atmospheric air packaging.

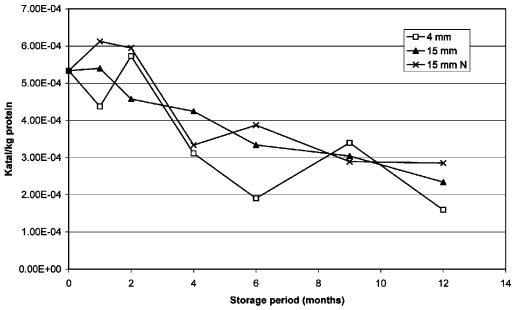

Figure 5. Specific activity of ADH analyzed in leek slices during frozen storage. See **Table 3** for specification of statistical differences within one treatment.

The production of ketones, alcohols, and furans are not affected by the slice thickness neither in the fresh leek slices nor after the frozen storage, as there are no statistical differences between 4- and 15-mm slices at each point of analysis. The effect of frozen storage is clear though, as all of the compounds except 2-pentyl furan increase during storage. Nitrogen packaging reduces this development during the storage, as these compounds are found in significantly lower concentrations than in the 15-mm slices.

Figures 3−**5** show the enzymatic activity of LOX, HPL, and ADH. LOX activity (**Figure 3**) decreases during the first two months, and after that, the activity is constant for 4- and 15-mm slices. There is no statistical difference between the two slice thicknesses, which indicates that the ability of LOX in the frozen tissue to gain activity when thawed is not influenced by slice thickness. After the drop in activity, 15-mm N increases slightly again, and apart from one inexplicable result from 15-mm N after 9 months of storage, which is higher than at the start, the activity ends up being significantly higher than the slices packed in atmospheric air. It is not obvious why the LOX activity is showing a maximum at 9 months of storage under nitrogen when tested under assay conditions. However, as the LOX reaction involves oxygen as the second substrate; a stabilizing influence of longer storage under nitrogen on the activity of LOX could possibly be anticipated.

HPL activity (**Figure 4**) is decreased during storage, reaching a minimum after 4−9 months of storage. HPL is not affected by nitrogen packaging, but slice thickness results in significantly higher activity after 12 months of frozen storage. ADH activity (**Figure 5**) decreases all through storage but is not significantly influenced by slice thickness or atmosphere after 12 months of frozen storage.

Most of the sulfur compounds decrease effectually during storage when packaged in atmospheric air, but all sulfur compounds are still present after 12 months of frozen storage. The 4-mm slices produce significantly most of nearly all of the sulfur compounds both in the fresh leek slices and after storage, but these slices also generate the largest concentration of aldehydes, which appears as off-flavor. Nitrogen packaging has a great influence on the keeping of aroma compounds of leek slices during frozen storage, as the 15-mm N almost maintains the aroma profile of the fresh leek slices. The content of sulfur compounds in the 15-mm N after 12 months of frozen storage is kept at almost the same level as the fresh leek slices, and the generation of aldehydes, ketones, and alcohols are reduced significantly compared to 4- and 15-mm stored for 12 months. Slice thickness does not influence the activity of LOX and ADH, but 4-mm slices result in higher activity of HPL than 15-mm slices do. Nitrogen packaging results in higher activity of LOX after 12 months of frozen storage compared to atmospheric air, while HPL and ADH are unaffected.

ABBREVIATIONS USED

LOX, lipoxygenase; HPL, hydroperoxide lyase; ADH, alcohol dehydrogenase; 15 mm N, 15 mm leek slices packed in nitrogen; 12M, 12 months of frozen storage

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Paper 4

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Determination of Odor Active Aroma Compounds in Freshly Cut Leek (*Allium ampeloprasum* Var. Bulga) and in Long-Term Stored Frozen Unblanched and Blanched Leek Slices by Gas Chromatography Olfactometry Analysis

GHITA STUDSGAARD NIELSEN* AND LEIF POLL

Department of Dairy and Food Science and LMC, Centre of Advanced Food Studies, The Royal Veterinary and Agricultural University, Rolighedsvej 30, 1958 Frederiksberg C, Denmark

The odor active compounds in freshly cut leek slices and in blanched and unblanched leek slices stored for 12 months were investigated by a detection frequency method. Fifteen judges were evaluating the three samples randomized. The most important aroma compounds in the freshly cut leek slices were dipropyl disulfide, methyl propenyl disulfide, pentanal, decanal, and propyl propenyl disulfide in order of priority. When stored frozen and unblanched for 12 months, the aroma composition changed and the most important compounds became pentanal, decanal, 2,5-dimethyl furan, unknown compound I, and dipropyl disulfide. Blanching before freezing prevented to some degree these changes but also reduced the perceived intensity of the aroma compounds. The most important aroma compounds in the blanched sample were dipropyl disulfide, unknown compound I, pentanal, 2,5-dimethyl furan, and propyl propenyl disulfide.

KEYWORDS: Leek; GC-O; aroma compounds; frozen storage; blanching

INTRODUCTION

The production of volatile compounds in freshly cut leek and other *Allium* species has given rise to many publications during the past decades, but few papers (*1*−*4*) deal with the GC-O analysis on leeks; consequently, the evaluation of the odor active compounds still remains relatively unsolved.

According to the literature, numerous sulfur-containing volatile compounds dominate the aroma profile of fresh leek. Sulfurous volatiles originate from the alliinase (EC 4.4.1.4) catalyzed decomposition of the odorless nonvolatile precursors *S*-alk(en)ylcysteine sulfoxides (*5*, *6*) The products, sulfenic acids, are highly reactive (*7*) and combine quickly to form thiosulfinates. Thiosulfinates are responsible for the odor of freshly cut leeks (*7*, *8*), but as they are relatively unstable (*5*, *9*), they rearrange to form polysulfides and thiosulfonates. As thiosulfonates are transformed to the corresponding monosulfide, the final products of the reaction are a combination of mono- and polysulfides with all of the possible combinations of the existing alk(en)yl radicals.

Products from the lipoxygenase (LOX) pathway are also found to be important for the leek aroma (*7*, *9*, *10*). LOX (EC 1.13.11.12) catalyzes the formation of hydroperoxy derivatives of polyunsaturated fatty acids with a *cis*,*cis*-pentadiene moiety (*11*, *12*) under the consumption of dioxygen. Volatile aldehydes are produced by the action of hydroperoxide lyase upon the formed hydroperoxides (*11*−*13*). All of these aldehydes, both saturated and nonsaturated, can be further metabolized by alcohol dehydrogenase (EC 1.1.1.1) to the corresponding alcohols (*12*). This formation of mainly aldehydes and alcohols may contribute to the aroma of freshly cut leeks, but it is not very pronounced in the fresh leek because of the pungency of thiosulfinates and thiopropanal-*S*-oxide (*9*). Previous investigations of unblanched frozen leek slices (*1*, *10*) have shown though that when leek slices are stored frozen for a longer period, the production of aldehydes probably has an influence on the aroma profile as well, both because of the increasing amount of these compounds and because of the diminishing content of sulfur compounds (*1*, *10*). Normally, manufacturing of frozen vegetables includes a blanching step to destroy the catalytic activity of enzymes and prevent this off-flavor formation. Leeks, however, are often processed without blanching, mainly because this has an undesirable effect on the texture. Consequently, enzymatic activity is still possible during frozen storage and in the thawing process.

Four papers have been found describing GC-O analysis on leek aroma. One paper is concerned with the determination of important aroma compounds from steam-distilled leek by means of GC-O (*3*); components contributing to the specific leek flavor were 1-propanethiol and 4 polysulfides. Another paper by the same authors (*4*) deals with GC-O analysis on headspace condensation extract of cut leeks collected for 24 h at 30 °C. This showed that besides the above-mentioned compounds five other polysulfides were important for the leek aroma. GC-O investigations on rehydrated dried leeks (*2*) also found that

* To whom correspondence should be addressed. Tel: +45-35283435. Fax: +45-35283265. E-mail: gni@kvl.dk.

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sulfur-containing components are the main odor active compounds in rehydrated leeks. A study (*1*) on GC-O analysis of unblanched leeks frozen for 3 months found that there is no key component in leeks but that both sulfur compounds and aldehydes contribute to the aroma.

The purpose of the present study was to evaluate both the odor active compounds in freshly cut leeks and the impact of the alteration of the aroma profile that occurs during frozen storage for 12 months of both blanched and unblanched samples.

MATERIALS AND METHODS

Plant Material. Leeks (*Allium ampeloprasum* var. Bulga) were harvested fully matured at Funen, Denmark, in October 2001 and immediately used for the experiment.

Chemicals. 1-Propanethiol, methyl propyl disulfide, dipropyl disulfide, dimethyl trisulfide, 2,5-dimethylthiophene, butanal, pentanal, hexanal, octanal, nonanal, decanal, (*E*)-2-hexenal, (*E*)-2-heptenal, (*E*)-2-octenal, 2-methyl-(*E*)-2-pentenal, (*E,E*)-2,4-heptadienal, 1-octen-3-ol, 1-octen-3-one, propanoic acid, and 4-methyl-1-pentanol were bought commercially from Sigma-Aldrich, Copenhagen, Denmark.

Sample Preparation. *Fresh Samples*. The white part of the leek stem was cleaned and cut into 15 mm slices immediately before the headspace collection of aroma compounds.

Unblanched Frozen Samples. The white part of the leek stem was cleaned and cut into 15 mm slices and frozen immediately in a blast freezer at −20 °C for 10 min, so that the center temperature reached −20 °C.

Blanched Frozen Samples. The white part of the leek stem was cleaned and cut into 15 mm slices (500 g) and immediately immersed in a pot with lid containing 1 L of boiling water; blanching time, 225 s. After the they were blanched, the leek slices were dripped off for 15 s, cooled in crushed ice for 15 min, and frozen in a blast freezer at −20 °C for 10 min, so that the center temperature reached −20 °C.

The frozen samples (unblanched and blanched) were packed in airtight glass jars with atmospheric air as the headspace and kept at −20 °C in the dark until analysis.

Experimental Design. Fresh slices were analyzed at harvest time, and subsequently, unblanched samples and blanched samples were analyzed after 12 months of storage. Each sample was analyzed for content of aroma compounds (for identification) in three replicates and GC-O analysis with 15 judges using the detection frequency method described by refs *14*−*16*.

Dynamic Headspace Collection of Aroma Compounds. Aroma compounds were isolated by dynamic headspace with nitrogen (purity = 99.8%) as the purge gas. Fresh or frozen leek slices (50 g) were crushed with 200 mL of tap water and 4 mL of internal standard (50 ppm of 4-methyl-1-pentanol in tap water) for 120 s in a Waring commercial blender. After the mixture had been transferred to a 1 L glass flask by adding another 150 mL of tap water, it was left for 10 min at 30 °C with agitation (200 rpm) to equilibrate the temperature before purging. Aroma compounds were trapped on 250 mg of Tenax GR (mesh size = 60/80, Buchem bv, Apeldoorn, The Nederlands) for 45 min with a nitrogen flow of 75 mL/min and agitation (200 rpm).

GC-MS Analysis. Desorption of aroma compounds was done thermally by an ATD 400 automatic thermal desorption system (Perkin-Elmer, Bucks, England). The desorption temperature of the trap to the cold trap (contains 30 mg of tenax GR; temperature, 5°C) was 250 °C for 15 min with a helium flow of 60 mL/min. The desorption temperature of the cold trap was 300 °C for 4 min with a helium flow of 31 mL/min and an outlet split ratio of 1:30. The temperature of the transfer line to the chromatograph was 175 °C. Separation was performed by a GC-MS (HP G1800 A GCD system) with the following conditions: column, DB Wax from J&W Scientific, CA (30 m × 0.25 mm i.d. × 0.25 μm film thickness); carrier gas, helium; start flow, 1 mL/min; split ratio, none; column pressure, 48 kPa (constant); oven program, 45 °C for 10 min, 3 °C/min to 240 °C, constant at 240 °C for 30 min; temperature of the interface to the MS, 250 °C. The mass selective detector was operated in the electron ionization mode (ionization energy, 70 eV), and the *m/z* (mass/charge) ratio ranged between 10 and 425. Identification was done by probability-based matching with mass spectra in the G1035A Wiley library (Hewlett-Packard). Identity was confirmed by checking with mass spectra and retention indices obtained in the laboratory from reference compounds for the following compounds: 1-propanethiol, methyl propyl disulfide, dipropyl disulfide, dimethyl trisulfide, 2,5-dimethyl thiophene, butanal, pentanal, hexanal, octanal, nonanal, decanal, (*E*)-2-hexenal, (*E*)-2-heptenal, (*E*)-2-octenal, 2-methyl-(*E*)-2-pentenal, (*E,E*)-2,4-heptadienal, 1-octen-3-ol, 1-octen-3-one, and propanoic acid. The retention time indices (RTI) of the volatile compounds were calculated with a mixture of hydrocarbons (C_9−C_{26}) as references according to the method of ref *17*.

GC-O and GC-FID Analysis. Desorption of aroma compounds was done thermally by a Short Path Thermal Desorption from Scientific Instrument Services Inc. Ringoes, NJ. The desorption temperature of the trap was 250 °C for 3 min with a helium flow of 60 mL/min. Separation was performed by a Hewlett-Packard 5890 GC with the following conditions: column, DB Wax from J&W Scientific (30 m × 0.25 mm i.d. × 0.25 μm film thickness); carrier gas, helium; start flow, 1 mL/min; split ratio, 1:20; column pressure, 88 kPa (constant); oven program, 45 °C for 10 min, 3 °C/min to 240 °C, constant at 240 °C for 30 min; detector temperature, 250 °C; air flow, 345 mL/min; hydrogen flow, 35 mL/min.

For the GC-O analysis, the FID detector was detached and the total flow was led to an olfactory detector outlet ODO-1 from SGE, Ringwood, Victoria, Australia. The flow from the olfactory outlet was combined with humidified air (150 mL/min) to avoid nasal dehydration. Fifteen untrained judges evaluated all three samples, and each sniffing session continued for 45 min. The judges were instructed to note start and finish time of the odor and a description of the odor. The odor descriptions were not checked by reference compounds. The judges were sniffing the samples randomized, and each judge was only sniffing one sample per day to avoid lassitude. The sniffing procedure was performed in a temperature-controlled room (20 °C).

Computation of Aromagrams. The 15 individual aromagrams of one sample were added up to one aromagram. The NIF value was calculated as the number of judges in percentage detecting the odor at the peak as described by refs *14* and *16*, and the SNIF value was calculated as the summed minutes that one peak lasted.

Identification of the Aromagrams. Identification of the detected odors was done by comparison of leek chromatograms obtained from GC-FID and GC-MS and by analyzing a known mixture of 12 aroma compounds by GC-FID, GC-MS, and GC-O and interpolating between compounds. The odor descriptions generated by the judges were not used for identification of the compound.

RESULTS AND DISCUSSION

Table 1 lists the odors that were detected during the GC-O analyses, and **Table 2** lists the 15 most important compounds in each of the three samples as evaluated by the largest NIF value and SNIF value (*14*). The judges were asked to define both a start and a finish time, but in some cases, there was no intermission between two odors; only the character of the odor changed. In that case, the judge was asked to state the same time as the finish time for the first odor and the start time for the next odor. For the fresh leek slices, this resulted in a level of four judges (26.7%) sniffing something all of the time starting approximately halfway through the sniffing time, and for that reason, a noise level of four judges was chosen. Many of the odors were very persistent late in the chromatogram, and it was hard for the judges to determine when the odor was over.

Twelve out of 43 compounds in **Table 1** are listed as unknown. This is either because there was no peak present in the MS chromatogram matching the time or that the MS software could not give certain identifications; in some cases though (unknowns A, B, D, G, and I), the MS software gave an indication of sulfur compound.

Table 1. Odors Detected by GC-O Analysis of Fresh Leek Slices and Blanched and Unblanched Leek Slices Stored Frozen for 12 Months

			0M U		12M U		12M B	
identification	RTI[a]	description of odor	NIF[b]	SNIF[c]	NIF	SNIF	NIF	SNIF
total minutes				183.4		168.0		121.6
		Sulfur Compounds						
1-propanethiol	843	fresh leek, fresh onion	60.0	4.9	40.0	2.7	40.0	2.7
methyl propyl disulfide	1217	rotten, fried onions, sour cabbage	60.0	5.0	53.3	2.2	40.0	1.6
methyl propenyl disulfide[d,e] no. 1	1250	fresh leek, strong chives	100.0	8.7	66.7	3.5	80.0	5.3
methyl propenyl disulfide[d,e] no. 2	1274	rotten water, rotten vegetables	80.0	4.6	46.7	1.6	46.7	1.7
methyl-2-propenyl disulfide[d]	1266	strong raw onion, leek	53.3	2.7	40.0	1.8		
dipropyl disulfide	1365	strong raw onion, sulfuric, fresh leek	100.0	9.5	93.3	7.7	100.0	9.0
propyl propenyl disulfide[d,e] no. 1	1393	fresh leek, bouillon	80.0	3.2	93.3	5.4	73.3	2.9
propyl propenyl disulfide[d,e] no. 2	1419	freshly cut leeks, strong, green	100.0	5.7	93.3	6.2	93.3	5.0
propyl-2-propenyl disulfide[d]	1410	heat treated leeks, leaves of leek	46.7	2.4	40.0	2.0		
dimethyl trisulfide	1356	solvent, rotten onion, tainted	93.3	3.4	73.3	1.9	73.3	2.0
diisopropyl trisulfide[d]	1646	sharp onion smell, chives	66.7	5.1	86.7	6.1	73.3	6.4
propyl propenyl trisulfide[d,e] no. 1	1743	sourish, solvent	86.7	4.5	93.3	3.0	73.3	5.5
propyl propenyl trisulfide[d,e] no. 2	1765	onion soup	66.7	4.3	60.0	3.0	33.3	1.2
2,5-dimethyl thiophene	1179	spring onions, fresh onion	33.3	2.4				
3,4- or 2,4-dimethyl thiophene[d]	1240	wood, dry smell, green, bookstore	80.0	4.9	53.3	2.5	33.3	2.3
total of sulfur compounds				71.3		49.6		45.6
		Aldehydes						
butanal	877	rotten, fried onions	33.3	1.3	60.0	2.3		
pentanal	976	fried onions, burned, boiled leek	100.0	7.7	100.0	8.0	100.0	5.4
hexanal	1070	green, grass	86.7	5.7	86.7	5.2	53.3	3.0
octanal	1279	orange, rubber			60.0	3.8	53.3	1.6
nonanal	1382	paint, turpentine	40.0	2.5	53.3	2.2	40.0	0.7
decanal	1486	cooking smell, sweat, refuse pail	100.0	6.0	100.0	7.3	66.7	3.0
(*E*)-2-hexanal	1201	fresh onion	40.0	3.3	60.0	4.5	33.3	1.0
(*E*)-2-heptenal	1306	forest, sweat	73.3	5.2	53.3	3.5	46.7	3.2
(*E*)-2-octenal	1412	soap, old raw leek, compost	66.7	2.7	80.0	2.6	40.0	1.5
(*E,E*)-2,4-heptadienal	1474	chemical leek smell, fried onion	73.3	3.3	60.0	3.3	60.0	2.3
2-ethyl-(*E*)-2-butenal[d]	1139	green leaves, fresh grass	73.3	4.4	53.3	4.8		
2-methyl-(*E*)-2-pentenal	1153	cabbage, paint, prickling	66.7	4.8	46.7	1.9		
total of aldehydes				46.9		49.4		21.7
		Miscellaneous						
2,5-dimethyl furan[d]	952	bouillon	100.0	4.5	100.0	5.5	100.0	4.1
1-octen-3-ol	1449	heat treated onion, burned	93.3	4.4	93.3	5.3	93.3	4.8
1-octen-3-one	1290	mushroom	73.3	2.4	80.0	3.4	73.3	3.3
propanoic acid	1528	ashtray, furniture depository	66.7	2.1	73.3	2.0	66.7	2.2
unknown A	1446	fried onion, unpleasant	66.7	1.9	60.0	2.1	33.3	1.1
unknown B	1468	fried onion, hazelnuts	93.3	7.9	93.3	7.6	80.0	6.9
unknown C	1495	fresh prickling onion, nettles, green	73.3	6.3	80.0	6.8	60.0	5.2
unknown D	1503	solvent, unpleasant	80.0	3.7	73.3	3.1	66.7	3.7
unknown E	1522	spring onion, red bell pepper	80.0	2.7	73.3	2.3	80.0	3.5
unknown F	1560	mild onion, sweet			73.3	6.5	46.7	2.2
unknown G	1566	sour–sweet sauce, mustard	80.0	9.6			53.3	3.3
unknown H	1595	roasted onions, hazelnuts			60.0	4.4	46.7	2.7
unknown I	1607	fresh, raw leek/onion	93.3	8.2	93.3	8.2	100.0	7.7
unknown J	1664	chemical, old onions	66.7	4.6	73.3	4.7	66.7	3.6
unknown K	1687	green, fresh			53.3	3.2		
unknown L	1702	sweetish leek, flower, green weeds	53.3	6.9	46.7	3.9		

[a] Retention time index. [b] Number of judges in percentage detecting the odor at the peak. [c] Total of minutes the odor is detected by all judges. [d] Compound was identified by GC-MS data alone. [e] *Z* and *E* isomers.

Numerous strong odors were present in the freshly cut leek slices, and many of these were sulfur compounds. The most important compound in this sample was dipropyl disulfide (detected by 100% of the judges with a SNIF value of 9.5 min), which in prior investigations was found to be the most abundant aroma compound in fresh leeks (*10*), but also, methyl propenyl disulfide (no. 1; see **Table 1**) (SNIF value, 8.7 min) and propyl propenyl disulfide (no. 2) (SNIF value, 5.7 min) were detected by all judges. Several other sulfur compounds were detected by more than two-thirds of the judges [3,4- or 2,4-dimethyl thiophene, methyl propenyl disulfide (no. 2), dimethyl trisulfide, propyl propenyl disulfide (no. 1), diisopropyl trisulfide, and propyl propenyl trisulfide (nos. 1 and 2)] indicating that sulfur compounds are important for the leek aroma. This was also found in headspace condensate of fresh leeks (*4*) and in rehydrated leeks (*2*). 1-Propanethiol, which other authors (*2*, *4*) found to be important in leeks, was detected by 60% of the judges with a SNIF value of 4.9 min, which does not suggest any major importance as it was not among the 15 most important odors (**Table 2**). No thiosulfinates were detected in the freshly cut leek slices; this is explained by the isolation method and the GC-MS method that most likely decomposes these compounds (*7*, *10*).

The odor description of the sulfur compounds was mainly given as fresh leek or onion, but some of them were also described as unpleasant; methyl propyl disulfide was described as rotten or sour cabbage; methyl propenyl disulfide (no. 2) was described as rotten water or rotten vegetables; dimethyl trisulfide was described as rotten onion or tainted; and propyl propenyl trisulfide (no. 1) was described as sourish or solvent.

Table 2. Fifteen Most Important[a] Odors Detected by GC-O Analysis of Fresh Leek Slices and Blanched and Unblanched Leek Slices Stored Frozen for 12 Months

	0M U			12M U			12M B		
no.	compound	NIF	SNIF	compound	NIF	SNIF	compound	NIF	SNIF
1	dipropyl disulfide	100.0	9.5	pentanal	100.0	8.0	dipropyl disulfide	100.0	9.0
2	methyl propenyl disulfide[b,c] no. 1	100.0	8.7	decanal	100.0	7.3	unknown I	100.0	7.7
3	pentanal	100.0	7.7	2,5-dimethyl furan[b]	100.0	5.5	pentanal	100.0	5.4
4	decanal	100.0	6.0	unknown I	93.3	8.2	2,5-dimethyl furan[b]	100.0	4.1
5	propyl propenyl disulfide[b,c] no. 2	100.0	5.7	dipropyl disulfide	93.3	7.7	propyl propenyl disulfide[b,c] no. 2	93.3	5.0
6	2,5-dimethyl furan[b]	100.0	4.5	unknown B	93.3	7.6	1-octen-3-ol	93.3	4.8
7	unknown I	93.3	8.2	propyl propenyl disulfide[b,c] no. 2	93.3	6.2	unknown B	80.0	6.9
8	unknown B	93.3	7.9	propyl propenyl disulfide[b,c] no. 1	93.3	5.4	methyl propenyl disulfide[b,c] no. 1	80.0	5.3
9	1-octen-3-ol	93.3	4.4	1-octen-3-ol	93.3	5.3	unknown E	80.0	3.5
10	dimethyl trisulfide	93.3	3.4	propyl propenyl trisulfide[b,c] no. 1	93.3	3.0	diisopropyl trisulfide[b]	73.3	6.4
11	hexanal	86.7	5.7	diisopropyl trisulfide[b]	86.7	6.1	propyl propenyl trisulfide[b,c] no. 1	73.3	5.5
12	propyl propenyl trisulfide[b,c] no. 1	86.7	4.5	hexanal	86.7	5.2	1-octen-3-one	73.3	3.3
13	unknown G	80.0	9.6	unknown C	80.0	6.8	propyl propenyl disulfide[b,c] no. 1	73.3	2.9
14	3,4- or 2,4-dimethyl thiophene[b]	80.0	4.9	1-octen-3-one	80.0	3.4	dimethyl trisulfide	73.3	2.0
15	methyl propenyl disulfide[b,c] no. 2	80.0	4.6	(*E*)-2-octenal	80.0	2.6	unknown D	66.7	3.7

[a] The importance was evaluated by the NIF value and if equal the SNIF value. [b] Compound was identified by GC-MS data alone. [c] *Z* and *E* isomers.

These compounds were detected by 80−93.3% of the judges for 3.4−4.6 min, meaning that they are important contributors to the aroma profile of fresh leeks.

Apart from sulfur compounds, a lot of other compounds, mostly aldehydes, were detected. Eight of the 12 detected aldehydes, both saturated, nonsaturated, and branched, were detected by two-thirds or more judges, and the total minutes of detection was relatively high in some cases. The most important nonsulfur compounds in the freshly cut leek slices were pentanal (SNIF value, 7.7 min), decanal (SNIF value, 6.0 min), and 2,5-dimethyl furan (SNIF value, 4.5 min), which were all detected by 100% of the judges.

The odor descriptions of the nonsulfur compounds found in freshly cut leeks were mainly off-flavor notes. Some of the aldehydes were described as fried onions or leeks [butanal, pentanal, and (*E*,*E*)-2,4-heptadienal], but although butanal and pentanal are described as pungent by ref *18*, these descriptions are probably due to odors lasting from a preeluting compound. (*E*)-2-Hexenal was described as fresh onion, and this compound was previously reported as being very important for the aroma of freshly cut leeks (*7*). In this study, (*E*)-2-hexenal was detected by 40% of the judges and the total minutes of detection were 3.3, which did not place this compound among the most important in the fresh leeks (**Table 2**).

When the leek slices had been stored frozen and unblanched for 12 months, most of the sulfur compounds decreased both in total minutes of detection and in number of judges agreeing on the odor. As no statistical analysis was possible, the following interpretation was done on the basis of simple comparison. The total minutes of detecting sulfur compounds decreases from 71.3 min in the freshly cut leeks to 49.6 min in the frozen stored leeks; see **Table 1**. Exceptions are propyl propenyl disulfide (nos. 1 and 2), diisopropyl-trisulfide, and propyl propenyl trisulfide (no. 1), which were at the same level as the fresh leeks or slightly increasing. Most aldehydes increased [butanal, pentanal, (*E*)-2-hexenal, octanal, (*E*)-2-octanal, and decanal], two were unaffected by the frozen storage (hexanal and nonanal) and two decreased [(*E*)-2-heptenal and (*E*,*E*)-2,4-heptadienal]. 2-Methyl-(*E*)-2-pentenal, which is one of the breakdown products from the lachrymatory factor thiopropanal-*S*-oxide, was reduced during frozen storage, the total minutes of detection was more than halved, and the percent of judges detecting it decreased from 66.7 to 46.7%.

These results indicate that frozen storage has a strong influence on the aroma profile of the leek slices as mainly the sulfur compounds decreased in importance and the aldehydes increased. This is consistent with what Petersen et al. (*1*) found in their study on frozen leek slices. **Table 2** shows that the order of the 15 most important compounds evaluated on the basis of NIF and SNIF values changed so that pentanal moved from the third position to first. Dipropyl disulfide, which was no. 1 in the fresh leek slices, decreased from 100% and 9.5 min to 93.3% and 7.7 min in the unblanched frozen leek slices placing that compound in the fifth position. Furthermore, the sulfur compounds dimethyl trisulfide, 3,4- or 2,4-dimethyl thiophene, methyl propenyl disulfide (no. 2), and the two unknown compounds G and D, which most likely also are sulfur compounds, were replaced on the list by only one sulfur compound, diisopropyl trisulfide, and four other compounds, 1-octen-3-one, (*E*)-2-octenal, and unknowns C and F.

The blanching procedure did to some degree prevent this altering of the aroma profile during frozen storage, as many of the sulfur compounds still constituted an important part of the aroma. Dipropyl disulfide was detected by 100% of the judges with a SNIF value of 9.0 min, which was very close to what was found for the fresh leek slices and which left that compound in the first position. There were minor displacements in the order of the compounds in **Table 2** though. Decanal, which had a high impact in freshly cut leeks and in frozen unblanched leeks and hexanal, was not among the 15 most important compounds

in the blanched leeks. Also, two sulfur compounds [methyl propenyl disulfide (no. 2) and 3,4- or 2,4-dimethyl thiophene] were missing on the list but two others [propyl propenyl disulfide (no. 1) and diisopropyl trisulfide] were added.

The blanching process influenced the total minutes of detection though as the blanched samples resulted all together in a SNIF value of 121.6 min as compared to 183.4 min for the fresh and 168.0 min for the unblanched stored for 12 months, which indicates that the blanched leek slices have a lower odor intensity. This was also expressed in the fact that three sulfur compounds and five other compounds found in the fresh or the unblanched frozen leek slices were not sniffed at all in the blanched sample. Still, the total SNIF value of sulfur compounds (**Table 1**) in the blanched samples was in the same level as the stored unblanched leeks slices. The top 15 compounds shown in **Table 2** also indicate that there are most powerful compounds in the freshly cut leek slices and the unblanched frozen stored leek slices, as compound no. 15 in these two cases was detected by 80% of the judges whereas it was 66.7% for the blanched leek slices. This shows that the blanching procedure has a reducing effect on production of both sulfur compounds and aldehydes, probably because of both leaching of volatiles to the blanching water during the blanching process (*19*) and by preventing catalytic activity of enzymes during the frozen storage.

In conclusion, six compounds [dipropyl disulfide, methyl propenyl disulfide (no. 1), pentanal, decanal, propyl propenyl disulfide (no. 2), and 2,5-dimethyl furan] were detected by 100% of the judges in the freshly cut leeks and four more (two unknowns, 1-octen-3-ol, and dimethyl trisulfide) were detected by 93.3% of the judges, which signifies that no single key component was present in leek. When stored frozen and unblanched for 12 months, the aroma in the leek slices changed toward less sulfur compounds being important, as the total SNIF value of sulfur compounds altered from 71.3 min in freshly cut leeks to 49.6 min in the frozen samples. At the same time, aldehydes were in equal level (46.9 min in the freshly cut leeks and 49.4 in the frozen stored), which means that the aroma profile changed. Blanching prior to frozen storage prevented this alteration to some degree as the ratio between sulfur compounds and aldehydes was almost the same as in the freshly cut leeks, but the total level of perceptible odors decreased.

ABBREVIATIONS USED

GC-O, GC−olfactometry; NIF, nasal impact frequency; SNIF, surface of nasal impact frequency; 0M U, freshly cut unblanched leek slices; 12M U, unblanched leek slices stored for 12 months; 12M B, blanched leek slices stored for 12 months.

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Paper 5

Nielsen G. S.; Larsen L. M.; Poll L. Formation of volatile compounds in model experiments with crude leek (*Allium ampeloprasum* Var. *Lancelot*) enzyme extract and linoleic acid or linolenic acid.

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Formation of Volatile Compounds in Model Experiments with Crude Leek (*Allium ampeloprasum* Var. *Lancelot*) Enzyme Extract and Linoleic Acid or Linolenic Acid

GHITA STUDSGAARD NIELSEN,*,† LONE MELCHIOR LARSEN,‡ AND LEIF POLL†

Department of Food Science and LMC, Centre of Advanced Food Studies, The Royal Veterinary and Agricultural University, Rolighedsvej 30, 1958 Frederiksberg C, Denmark and Chemistry Department, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark

Three continuous assays are described for lipoxygenase (LOX), hydroperoxide lyase (HPL) and alcohol dehydrogenase (ADH) in leek tissue. The catalytic activity of LOX showed significant difference (significance level 5%) between linolenic acid (9.43×10^{-4} katals per kg protein) and linoleic acid (2.53×10^{-4} katals per kg protein), and the pH-optimum of LOX was 4.5−5.5 against linoleic acid. The catalytic activity of HPL was statistically the same for 9-(S)-hydroperoxy-(10E,12Z)-octadecadienoic acid (1.01×10^{-2} katals per kg protein) and 13-(S)-hydroperoxy-(9Z,11E)-octadecadienoic acid (7.69×10^{-3} katals per kg protein). ADH showed a catalytic activity of 5.01×10^{-4} katals/kg of protein toward hexanal. Model experiments with crude enzyme extract from leek mixed with linoleic acid or linolenic acid demonstrated differences in the amount of produced aroma compounds. Linoleic acid resulted in significantly most hexanal, heptanal, (*E*)-2-heptenal, (*E*)-2-octenal, (*E,E*)-2,4-decadienal, pentanol, and hexanol, whereas linolenic acid resulted in significantly most (*E*)-2-pentenal, (*E*)-2-hexenal, (*E,Z*)-2,4-heptadienal, (*E,E*)-2,4-heptadienal, and butanol. Leek LOX produced only the 13-hydroperoxide of linoleic acid and linolenic acid.

KEYWORDS: Leek; flavor; lipoxygenase; hydroperoxide lyase; alcohol dehydrogenase; enzyme assays

INTRODUCTION

The aroma of freshly cut leek and other *Allium* species is dominated by numerous sulfur containing volatile compounds originating from decomposition of the odorless nonvolatile precursors S-alk(en)yl-cysteine sulfoxides by action of alliinase (EC 4.4.1.4) (*1*, *2*). However, volatiles produced by the lipoxygenase pathway also contribute to the aroma profile of freshly cut leeks (*3*) and especially of frozen stored leek slices (*4*). The lipoxygenase pathway in plants is very complex and over hundred products from lipoxygenase-generated hydroperoxides of linoleic acid have been reported (*5*). In the plant the catalyzed reactions are involved in defense, senescence, seed germination, stress response, and communication (*6*−*8*), and some of these reactions also lead to the formation of short-chain aldehydes and alcohols, which will contribute to the aroma of freshly cut leeks. Production of these aroma compounds could also be possible by autoxidation (*9*).

Lipoxygenases (EC 1.13.11.12) (LOX) are non-heme, iron-containing dioxygenases that catalyze the formation of hydroperoxy derivatives of polyunsaturated fatty acids with a *cis*,*cis*-pentadiene moiety (*7*, *10*) In plants, the most common substrates are linoleic acid and linolenic acid (*7*). Dioxygen is introduced either at the 9- or 13-carbon of linoleic or linolenic acid leading to the formation of the corresponding conjugated hydroperoxy-diene or -triene fatty acid. The ratio of 9- to 13-hydroperoxides is depending on the origin of LOX (*5*, *7*).

Volatile aldehydes and nonvolatile oxoacids are produced by the action of hydroperoxide lyase (HPL) on the formed hydroperoxides (*7*, *11*). Likewise with LOX, there are different types of HPL classified according to their substrate specificity (*5*, *12*, *13*). One isoenzyme is specific toward the 9-hydroperoxide, one toward the 13-hydroperoxide and one is nonspecific and therefore cleaving both hydroperoxides (*13*). When metabolizing linolenic acid hydroperoxide, HPL retains the *Z*-configuration of the double bond from the hydroperoxide to the aldehyde, but isomerization by isomerases to the *E*-isomer occurs after the cleavage reaction (*12*). All of these aldehydes, both saturated and nonsaturated, can be further metabolized by alcohol dehydrogenase (EC 1.1.1.1) (ADH) to the corresponding alcohols by consumption of NADH (*11*).

Few investigations have been carried out on LOX, HPL, or ADH originating from leek or other *Allium* and therefore little is known about the potential substrate specificity for LOX and HPL in leek. Clayes et al. (*14*) have found lipoxygenase metabolites in onions, but they did not investigate the activity of lipoxygenase. Investigations have been made on the LOX

* To whom correspondence should be addressed. Tel.: +45-35283435. Fax: +45-35283265. E-mail gni@kvl.dk.
† Department of Dairy and Food Science and LMC.
‡ Chemistry Department.

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activity in leek (*4*, *15*, *16*), onions and garlic (*17*), and chives (*18*), but only refs *4* and *16* were related to aroma formation.

The aim of this paper was to develop and/or optimize three continuous assays for analyzing activities of LOX, HPL, and ADH in leek. One assay was based on measuring oxygen consumption and two assays were based on the oxidation of NADH measured spectrophotometrically. Also the nature of LOX and HPL originating from leek was investigated with reference to substrate specificity and determination of pH-optimum of LOX. Finally, this paper looks into the formation of aroma compounds when crude enzyme extract from leek are mixed with linoleic acid or linolenic acid.

MATERIALS AND METHODS

Plant Material. Leeks (*Allium ampeloprasum*, Var. *Bulga*) were harvested fully matured at Funen, Denmark in October 2001 and used for the optimization of the enzyme assays immediately after. Leeks (*Allium ampeloprasum*, Var. *Lancelot*) were bought in a local store and used for the aroma model experiment immediately after.

Chemicals. Reference compounds (purity ≥ 97%): Propanal, butanal, pentanal, hexanal, heptanal, octanal, nonanal, (*E*)-2-butenal, (*E*)-2-pentenal, (*E*)-2-hexenal, (*E*)-2-heptenal, (*E*)-2-octenal, (*E*,*E*)-2,4-heptadienal, (*E*,*E*)-2,4-decadienal, 1-propanol, 1-butanol, 1-pentanol, and 1-hexanol were bought commercially from Sigma-Aldrich, Copenhagen, Denmark. (*E*,*Z*)-2,4-Heptadienal was occurring as an impurity in (*E*,*E*)-2,4-heptadienal. All other chemicals, except 9-(S)-hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid, were of analytical grade and were bought commercially from Sigma-Aldrich.

Synthesis of 9-(S)-Hydroperoxy-(10*E*,12*Z*)-octadecadienoic Acid (9-HPODE). Sodium linoleate (1.25 mL, 10mM), 4.75 mL of air saturated 50 mM potassium phosphate buffer (pH 7.0) and 1.50 mL of potato tuber lipoxygenase solution were mixed then agitated and purged with oxygen for 30 min. The solution was checked for absorbance at 234 nm to determine the content of 9-HPODE. According to Galliard and Phillips, (*19*) potato tuber LOX almost exclusively converts linoleic acid into 9-HPODE.

Preparation of Potato Tuber Lipoxygenase Solution for Synthesis. Grated potato tubers (50 g) and 50 mL of tap water were homogenized for 30 s in a Waring commercial blender and filtrated through a paper filter. A 2-mL aliquot of the liquid was applied to a DEAD-Cellulose C545 column (anion exchanger) followed by 6.0 mL of 50 mM potassium phosphate buffer (pH 7.0). Two fractions of 4.0 mL were collected; both were checked for activity, and the second one was used as the lipoxygenase solution.

Preparation of Enzyme Extract. The white part of the leek stem was cut into 4-mm slices. Enzyme extract was made by mixing 200 g of leek slices with 200 mL of potassium phosphate buffer (50 mM, pH 7.0 added 0.1% Triton X-100) for 45 s in a Waring commercial blender. The slurry was kept on ice with agitation for 30 min (100 rpm) and afterward filtered through a paper filter until 75.0 mL was collected. Seven of these extractions were mixed, frozen, and used as the enzyme source through all experiments.

Experimental Design. Crude enzyme extract from leeks was analyzed for catalytic activity of LOX, HPL, and ADH in triplicate. Substrate specificity of LOX toward linoleic acid and linolenic acid was investigated, and the pH-optimum was determined. Also, substrate specificity of HPL toward 9-(S)-hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid (9-HPODE) and 13-(S)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid (13-HPODE) was investigated.

The enzyme extract was mixed with linoleic acid or linolenic acid or phosphate buffer (blank samples) and left for 2, 10, 30, or 60 min to react prior to aroma analysis. Blank samples were also run on the fatty acids alone with a reaction time of 60 min.

Lipoxygenase Assay. LOX was assayed in a continuous assay by measuring consumption of initial dioxygen by an YSI 5100 dissolved oxygen meter (YSI Inc., Yellow Springs, OH) at 30 °C, using linoleic acid or linolenic acid as the other substrate. Calibration was done at 30 °C by air-saturated tempered phosphate buffer (0.2 M, pH 6.0) (21% dissolved dioxygen) followed by addition of sodium dithionite (0% dissolved dioxygen). To determine lipoxygenase activity, 27.2 mL of air-saturated phosphate buffer (0.2 M, pH = 6.0) and 3.9 mL of enzyme extract was mixed in a 33-mL conical flask and the measuring was started. After 30 s, the reaction was initiated by adding 1.9 mL of sodium linoleate solution (10 mM). Dioxygen was monitored over 20 min, measuring dioxygen content every second.

Blank samples were run on buffer added substrate or enzyme extract.

Calculation of LOX Activity. LOX activity was calculated as katals per kg of protein, and katals was defined as moles of O_2 consumed per second. This was done under the assumption that air-saturated phosphate buffer (0.2 M, pH 6.0) at 30 °C with a salt strength of 12.40 g/L contains 7.11 mg O_2/L(*20*). To determine the activity, the dioxygen level (millimoles of O_2) was plotted against time, and the steepest slope (millimoles of O_2 per second) on the curve was found using continuous linear regression over 20 s at a time.

Determination of pH-Optimum of LOX. pH-Optimum was examined by triplicate determinations of the catalytic activity of LOX against linoleic acid in 0.1 M acetate buffer with the following pH values: 3.5, 4.0, 4.5, 5.0, and 5.5 and in 0.2 M phosphate buffers with the following pH values: 6.0, 6.5, 7.0, and 7.5. Blank samples were run at each pH value.

Hydroperoxide Lyase Assay. HPL was assayed in a continuous coupled assay modified after (*21*). ADH converts the products of HPL's reaction on hydroperoxides, aldehydes, under the oxidation of NADH, which can be monitored spectrophotometrically at 340 nm.

Phosphate buffer (1.8 mL, 0.2 M, pH = 6.0), 300 μL of 2.2 mM NADH, 200 μL of ADH (150 units in 100 μL) and 300 μL of enzyme extract (filtrated through 1.2-μm and 0.45-μm filters from Orange Scientific, Braine-l'Alleud, Belgium) were transferred to a thermostatic (30 °C) 1-cm cuvette with a magnetic stirrer, and the measuring was started. After 30 s, 400 μl of 9-HPODE or 13-HPODE was added. The reaction was monitored over 600 s measuring every second.

Blank samples were run on buffer, NADH, and ADH with substrate or enzyme extract.

Calculation of HPL Activity. HPL activity was calculated as katals per kg of protein, and katals was defined as moles of hydroperoxide consumed per second. To determine the activity, the absorbance was plotted against time, and the steepest slope (absorbance/s) on the curve was found using continuous linear regression over 5 s at a time. Absorbance was converted to moles of hydroperoxide by the assumption that each mole of hydroperoxide metabolized produced one mole of aldehyde. Aldehydes are converted by alcohol dehydrogenase by the consumption of NADH 1:1. ϵ of NADH at 340 nm is 6220 L/mol • cm (*21*).

Alcohol Dehydrogenase Assay. ADH was assayed in a continuous assay by following the oxidation of NADH spectrophotometrically at 340 nm when hexanal was added.

Borate buffer (1.4 mL, 0.05 M, pH = 9.0), 300 μl of 2.2 mM NADH and 1000 μL of enzyme extract (filtrated through 1.2-μm and 0.45-μm filters from Orange Scientific) were transferred to a thermostatic (30 °C) 1-cm cuvette with a magnetic stirrer, and the measuring was started. After 30 s, 300 μL of hexanal (100 ppm) was added. The reaction was monitored over 600 s measuring every second.

Blank samples were run on buffer and NADH with substrate or enzyme extract.

Calculation of ADH Activity. Alcohol dehydrogenase activity was calculated as katals per kg of protein, and katals was defined as moles of hexanal consumed per second. Moles of hexanal were calculated on the basis of NADH under the assumption that hexanal and NADH were metabolized 1:1 and by the conditions described under calculation of HPL activity.

Determination of Protein Content. Protein content in the enzyme extraction was determined by Coomassie brilliant blue, according to the method described in US/EG Bulletin 1069 from Bio-Rad Life Science Group, Hercules, CA, using lyophilized bovine serum albumin as the standard protein.

Model Experiments. Air-saturated phosphate buffer (60.0 mL, 0.2 M, pH 6.0), 8.0 mL of enzyme extract and 4.0 mL of sodium linoleate solution (10 mM) or sodium linolenate solution (10 mM) were mixed in a closed 500-mL glass flask at 30 °C with agitation (200 rpm) for 2, 10, 30, or 60 min. Immediately after the reaction period, 3.0 g of

Table 1. Catalytic Activities of Lipoxygenase, Hydroperoxide Lyase, and Alcohol Dehydrogenase in Crude Enzyme Extract of Leek

substrate	lipoxygenase[a]	hydroperoxide lyase[a]	alcohol dehydrogenase[a]
linoleic acid	0.000943 ± 0.00026		
linolenic acid	0.000253 ± 0.000052		
9-HPODE[b]		0.0101 ± 0.0036	
13-HPODE[c]		0.00769 ± 0.0022	
hexanal			0.000501 ± 0.000093

[a] All values are in katal/kg protein. [b] 9-(S)-Hydroperoxy-(10E,12Z)-octadecadienoic acid. [c] 13-(S)-Hydroperoxy-(9Z,11E)-octadecadienoic acid.

calcium chloride was added with agitation to inactivate enzymes. Aroma analysis was performed directly after the saturation.

Blank samples were made with enzyme extract with 4 mL of phosphate buffer instead of the fatty acids or with each of the fatty acids with 8 mL of phosphate buffer instead of the enzyme extract. Blank samples were treated as described above, but only a reaction time of 60 min was applied.

Dynamic Headspace Analysis. Aroma compounds were isolated by dynamic headspace with nitrogen (purity = 99.8%) as purge gas. Internal standard (1.0 mL, 50 ppm 4-methyl-1-pentanol in tap water) was added to the solution in the 500-mL glass flask, which was left for 5 min at 30 °C with agitation (200 rpm) to equilibrate the temperature before purging. Aroma compounds were trapped on 250 mg Tenax GR (mesh size = 60/80, Buchem bv, Apeldoorn, The Nederlands) for 45 min with a nitrogen flow of 100 mL/min and agitation (200 rpm).

Desorption of aroma compounds was done thermally by an ATD 400 automatic thermal desorption system (Perkin-Elmer, England). Desorption temperature of the trap to the cold trap (contains 30 mg Tenax GR, temp 5 °C) was 250 °C for 15 min with a helium flow of 60 mL/min. Desorption temperature of the cold trap was 300 °C for 4 min, with a helium flow of 11 mL/min and an outlet split ratio of 1:10. Separation was performed by a GC-MS (HP G1800 A GCD system) with the following conditions: column, DB Wax from J&W Scientific (30-m × 0.25-mm i.d. × 0.25-μm film thickness); carrier gas, helium; start flow, 1 mL/min; split ratio, none; column pressure, 48 kPa (constant); oven program, 45 °C for 10 min, 6 °C/min to 240 °C, constant at 240 °C for 30 min. The mass selective detector was operated in the electron ionization mode (ionization energy, 70 eV), and the *m/z* (mass/charge) ratio ranged between 10 and 450. Identification was done by probability-based matching with mass spectra in the G1035A Wiley library (Hewlett-Packard). Identity was confirmed for all detected compounds by checking with mass spectra and retention indices obtained in the laboratory from reference compounds.

Quantification. Aliquots of 10 μL of the reference compounds propanal, butanal, pentanal, hexanal, heptanal, octanal, nonanal, (*E*)-2-butenal, (*E*)-2-pentenal, (*E*)-2-hexenal, (*E*)-2-heptenal, (*E*)-2-octenal, (*E,E*)-2,4-heptadienal, (*E,E*)-2,4-decadienal, 1-propanol, 1-butanol, 1-pentanol, and 1-hexanol were each dissolved in 20 mL of 96% ethanol and diluted with tap water into three appropriate concentrations depending on the concentration of the compounds in the model experiments. Dynamic headspace analysis was performed on 72.0 mL of the solutions with 1.0 mL of internal standard (50 ppm 4-methyl-1-pentanol in tap water) with the same flow, time, and temperature conditions as applied to the model experiments. Each reference compound was analyzed in triplicate in all three concentrations. The obtained peak area divided by the peak area of the internal standard was used to calculate the concentration of the same compound in the model experiments from the peak area of the compound divided by the peak area of the internal standard.

The retention time indices (RTI) of the volatile compounds were calculated with a mixture of hydrocarbons (C_9−C_{26}) as references, according to the method of (*22*).

Statistical Analysis. Analysis of variance was carried out on each of the aroma compounds using the one-way and the factorial ANOVA procedure in the Analyst part of SAS, version 8.2, SAS institute Inc., Cary, NC. The effect of the reaction period and the type of substrate was tested. Sample means were compared by Duncan's multiple range test. A significance level of 5% was applied. The effect of pH on LOX activity and type of substrate on LOX and HPL was tested by the one-way ANOVA procedure and by Duncan's multiple range test (significance level 5%).

RESULTS AND DISCUSSION

To find an extraction method suitable for all three enzymes, extractions were made with phosphate buffer (0.2 M, pH = 6.0) added 0.1% Triton X-100, with acetate buffer (0.1 M, pH = 6.0), with tap water, or with 0.1 M $CaCl_2$ solution because of the possible calcium requirements of LOX isoenzymes. HPL is difficult to isolate because of its binding to membranes (*5*, *23*), probably to the thylakoid membrane of chloroplasts (*24*), and a detergent might be needed for the solubilization (*25*). Also, the effect of the extraction time was tested between 0 and 60 min. These experiments demonstrated that the most suitable method all together was to use phosphate buffer (0.2 M, pH = 6.0) with 0.1% Triton X-100 as the extraction fluid and to apply an extraction time of 30 min.

Table 1 shows the catalytic activities of LOX, HPL, and ADH measured in the crude enzyme extract. Measurements of LOX activity on the two fatty acids proved a significant difference, linoleic acid resulted in almost 4 times higher activity than linolenic acid. HPL activity was also checked on two substrates, the 9-hydroperoxide and the 13-hydroperoxide of linoleic acid. In most plants, HPL is specific for one of the hydroperoxides (*26*), but in this case, there was no statistical difference between the two substrates. This indicated that HPL from leek was capable of transforming both hydroperoxides or that two or three HPL isoenzymes were present. Husson and Belin (*26*) found two isoforms of HPL in green bell pepper but did not investigate the activity toward 9-hydroperoxides. The activity of ADH was tested with hexanal as substrate and was found to be 5.01 × 10^{-4} katals/kg of protein.

The pH-optimum of LOX in leek was 4.5−5.5 with linoleic acid as substrate (**Figure 1**). pH-Optimum with linolenic acid as substrate was not examined. We have not found any other investigations relating to pH-optimum of LOX in leeks, but Egert and Tevini (*18*) found that LOX activity in chives has a pH-optimum at pH 6, and investigations on potato LOX demonstrated pH-optimum at pH 5.5−6.0 (*27*,*28*). We found that the length of the lag phase was not influenced by the pH change (data not shown); the values were 50−150 s (not statistically different) but with no correlation to the pH change.

Table 2 displays all of the aldehydes and alcohols found in the model experiments of the two fatty acids, the enzyme extract without fatty acid added (blank), and the blank measurements of linoleic acid and linolenic acid after a reaction time of 60 min (autoxidation controls). Many sulfur compounds were detected as well, but as they are not relevant for the concern of this paper, the results were not shown or discussed in details. One thiol (1-propanethiol) was detected in minor concentrations in the enzyme extract, and although thiols can react with the carbonyl group in the aldehydes, this would occur in equal amounts in the three samples with enzyme extract and consequently not influence the result.

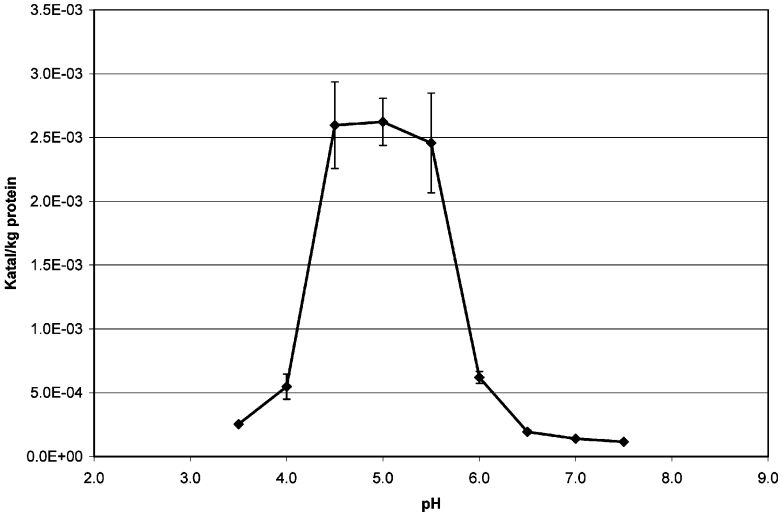

Figure 1. pH response curve for LOX activity against linoleic acid substrate in crude leek extract. Vertical bars indicate standard deviation.

Table 2. Aldehydes and Alcohols Found in the Model Experiments of the Leek Enzyme Extract with Either Linoleic Acid, Linolenic Acid, or No Fatty Acid (Enzyme Blank), or Fatty Acid without Leek Enzyme Extract (Acid Blanks) after a Reaction Time of 60 min

compound	RTI[a]	linoleic acid + enzymes[b]	linolenic acid + enzymes[b]	enzyme blank[b]	linoleic acid blank[b]	linolenic acid blank[b]
aldehydes						
propanal	762	0.838 ± 0.12	0.916 ± 0.057	0.761 ± 0.22	0.00 ± 0	0.147 ± 0.0057
butanal	867	0.00463 ± 0.00016	0.00459 ± 0.00037	0.00165 ± 0.00028	0.000293 ± 0.000027	0.00251 ± 0.00029
pentanal	984	0.162 ± 0.015	0.163 ± 0.0084	0.185 ± 0.021	0.00 ± 0	0.0177 ± 0.00090
hexanal	1111	0.895 ± 0.025	0.0721 ± 0.0045	0.130 ± 0.0072	0.00482 ± 0.00058	0.00 ± 0
heptanal	1197	0.00554 ± 0.00012	0.00232 ± 0.00025	0.00378 ± 0.00076	0.000688 ± 0.00016	0.00107 ± 0.00026
octanal	1299	0.000332 ± 0.0000044	0.000525 ± 0.000052	0.000808 ± 0.000031	0.000242 ± 0.000123	0.000409 ± 0.000040
nonanal	1398	0.000900 ± 0.00011	0.00164 ± 0.00026	0.00288 ± 0.000094	0.000258 ± 0.000027	0.000300 ± 0.000080
(*E*)–2-butenal	1046	0.00238 ± 0.00018	0.00995 ± 0.00048	0.0150 ± 0.0007	0.00 ± 0	0.00 ± 0
(*E*)–2-pentenal	1147	0.0161 ± 0.0011	0.0909 ± 0.00015	0.0236 ± 0.00070	0.00 ± 0	0.00 ± 0
(*E*)–2-hexenal	1230	0.00446 ± 0.000087	0.0073 ± 0.0010	0.00230 ± 0.000072	0.00 ± 0	0.00 ± 0
(*E*)–2-heptenal	1334	0.0384 ± 0.0012	0.0024 ± 0.00014	0.00438 ± 0.000029	0.00 ± 0	0.00 ± 0
(*E*)–2-octenal	1434	0.0345 ± 0.0014	0.00105 ± 0.00012	0.00191 ± 0.00094	0.00 ± 0	0.00 ± 0
(*E,Z*)–2,4-heptadienal	1464	0.00339 ± 0.00063	0.0151 ± 0.0022	0.00 ± 0	0.00 ± 0	0.00 ± 0
(*E,E*)–2,4-heptadienal	1490	0.00363 ± 0.00023	0.0118 ± 0.0011	0.000914 ± 0.000081	0.00 ± 0	0.00 ± 0
(*E,E*)–2,4-decadienal	1789	0.0575 ± 0.0029	0.00 ± 0	0.00 ± 0	0.00 ± 0	0.00 ± 0
alcohols						
1-propanol	1049	0.0156 ± 0.0019	0.0176 ± 0.0023	0.0147 ± 0.0014	0.00 ± 0	0.00 ± 0
1-butanol	1166	0.000959 ± 0.000025	0.00401 ± 0.000071	0.00142 ± 0.00012	0.00 ± 0	0.00 ± 0
1-pentanol	1274	0.0339 ± 0.00074	0.0108 ± 0.00031	0.0125 ± 0.00160	0.00 ± 0	0.00 ± 0
1-hexanol	1371	0.00640 ± 0.00053	0.00256 ± 0.000077	0.00331 ± 0.00014	0.00 ± 0	0.00 ± 0

[a] Retention time index. [b] All values except RTI are in mg/L ± standard deviation.

When the amount of aldehydes and alcohols obtained from enzymatic treatment of the two fatty acids after a reaction time of 60 min. was compared with the results from the enzyme extract blank (**Table 2**), there were evident differences. Linoleic acid led to formation of significantly mostly hexanal, heptanal, (*E*)-2-heptenal, (*E*)-2-octenal, (*E,E*)-2,4-decadienal, pentanol, and hexanol, whereas linolenic acid resulted in significantly mostly (*E*)-2-pentenal, (*E*)-2-hexenal, (*E,Z*)-2,4-heptadienal, (*E,E*)-2,4-heptadienal, and butanol. Butanal was produced in equal amounts in the two fatty acid samples but in higher concentration than the enzyme blank sample, whereas propanal, pentanal, and 1-propanol were produced in the same quantities in the two fatty acid samples and the enzyme blank sample. Octanal, nonanal, and (*E*)-2-butenal were significantly highest in the enzyme blank sample.

As seen in **Table 2,** most of the volatile compounds were not present in the linoleic acid blank and the linolenic acid blank samples; exceptions were butanal, hexanal, heptanal, octanal, and nonanal in linoleic acid and propanal, butanal, pentanal, heptanal, octanal, and nonanal in linolenic acid. However, these were produced in a significantly lower level than they were in the samples with the fatty acids added enzymes.

According to Hornostaj and Robinson (*13*) the cleavage of the 9-hydroperoxides of linoleic acid and linolenic acid by HPL are converted to (*Z*)-3-nonenal and (*Z,Z*)-3,6-nonadienal, respectively, whereas the cleavage of the 13-hydroperoxide yields hexanal and (*Z*)-3-hexenal, respectively. These aldehydes are further converted by the action of ADH into the corresponding alcohols or by isomerization of the (*Z*)-3-enals to (*E*)-2-enals by isomerases (*10*). Hexanal (**Figure 2**) was developing all

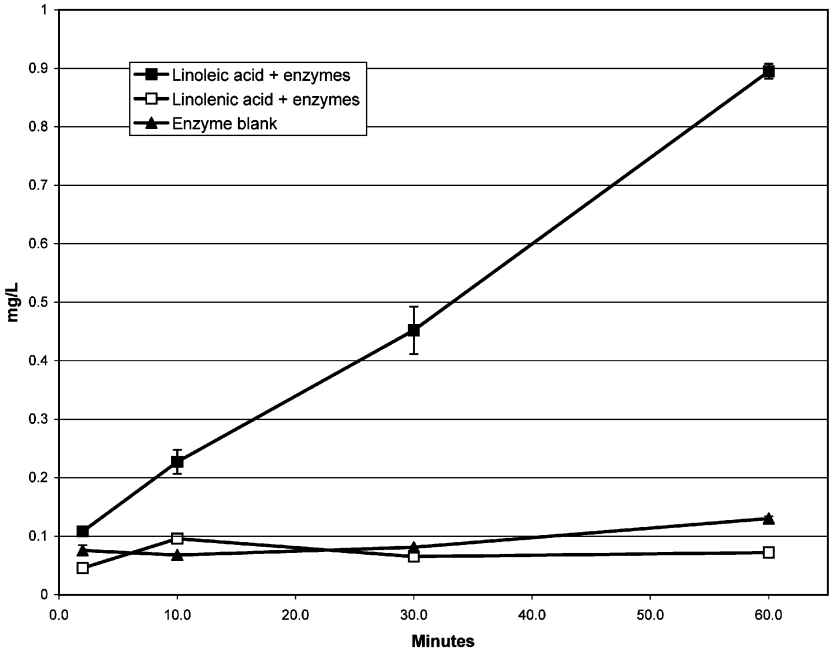

Figure 2. Development of hexanal during 60 min reaction in the model experiments of crude leek enzyme extract with linoleic acid or linolenic acid as substrate or without added fatty acid (enzyme blank). Vertical bars indicate standard deviation.

through the reaction time of 60 min when linoleic acid substrate was added, whereas the blank samples and the linolenic acid substrate showed no development over time, which confirmed the fact that hexanal is produced from the 13-hydroperoxide of linoleic acid (*29*). (*E*)-2-hexenal, which originates from the 13-hydroperoxide of linolenic acid, also showed statistical differences between the two fatty acids and the blank samples when the whole time range was considered (data not shown). In this case, linolenic acid resulted in the highest production of (*E*)-2-hexenal, but linoleic acid also showed an increase in (*E*)-2-hexenal compared to the enzyme blank. (*E*)-2-pentenal also showed the pattern described for (*E*)-2-hexenal, except that adding linoleic acid gave only a minor production of (*E*)-2-pentenal. This is consistent with Gardner et al. (*30*), who describe the formation of (*E*)-2-pentenal from the 13-hydroperoxide of linolenic acid by the action of LOX and ADH in soybean. (*E*,*E*)-2,4-Heptadienal and (*E*,*Z*)-2,4-heptadienal showed the same development.

The present aroma results indicated that only the 13-hydroperoxide of linoleic acid was produced, because only hexanal and not (*E*)-2-nonenal was found when linoleic acid was added. Likewise, only (*E*)-2-hexenal and not 3,6-nonadienal was found when linolenic acid was added. According to Gardner (*5*), LOX with pH-optimum near neutrality is normally specific toward 9-(S)-oxidations, while pH-optimum close to 9 usually results in specificity toward 13-(S)-oxidations. Our results could not confirm this, as pH-optimum was determined to be 4.5−5.5. The results of the aroma analysis also indicated that LOX was metabolizing linoleic acid more readily than linolenic acid, because production of hexanal when linoleic acid was added was more than 100 times greater than the production of (*E*)-2-hexenal when linoleic acid was added. The same trend was found for the catalytic activity of LOX expressed as moles of O_2 consumed per second toward the two substrates.

(*E*)-2-Heptenal and (*E*,*E*)-2,4-decadienal (**Figure 3**) showed the same development as hexanal, except that (*E*,*E*)-2,4-decadienal was not detected in the linolenic acid sample and the three blank samples. This indicated that the production was caused by enzymatic activity and not by autoxidation.

The addition of linoleic acid to the enzyme extract led to formation of approximate 30 times more (*E*)-2-octenal than when linolenic acid was added or with no fatty acid added. According to Rosahl (*7*), dioxygen is introduced either at the 9- or 13-carbon by LOX, which means that (*E*)-2-octenal is not a direct product of the lipoxygenase pathway. However, Haslbeck and Grosch (*31*) found that soybean lipoxygenase is also capable of producing the 8-, 10-, 12-, and 14-HPODE as minor components. (*E*)-2-Octenal was formed when the hydroperoxide was placed on the C-10 position of linoleic acid or by autoxidation of (*E*,*E*)-2,4-decadienal (*9*), and experiments with pea LOX showed that (*E*)-2-octenal was formed when linoleic acid was oxidized by LOX (*32*).

Propanal, pentanal, and 1-propanol were produced in statistically equal amounts in the three solutions with enzyme when looking at the whole lapse of time. These apparently did not originate from an enzymatic reaction on one of the two fatty acids and probably not from autoxidation either, because these compounds were not detected at all in the linoleic acid blank and in only minor concentrations (only propanal and pentanal) in the linolenic acid blank. Their origin was most likely from other enzymatic reactions and substrates present in the crude leek extract. Propanal is a breakdown product from the lachrymatory factor thiopropanal-S-oxide originating from alliinase activity (*33*) and 1-propanol can be a result of ADH's action on propanal. Formation of these three aroma compounds had probably already occurred during the preparation of the enzyme extract because of the available substrate in the extract

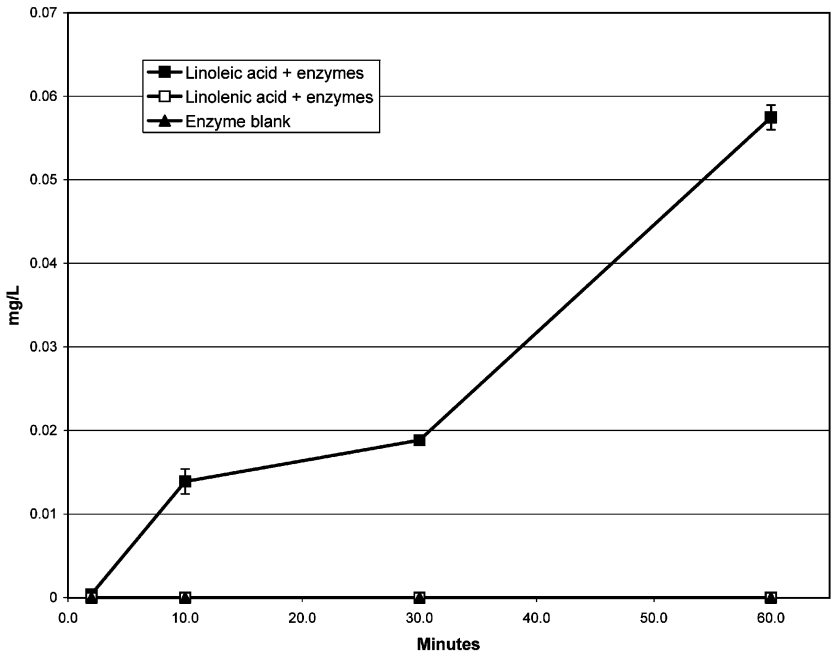

Figure 3. Development of (*E*,*E*)-2,4-decadienal during 60 min reaction in the model experiments of crude leek enzyme extract with linoleic acid or linolenic acid as substrate or without added fatty acid (enzyme blank). Vertical bars indicate standard deviation.

when the cells were ruined, as no further development over the 60 min was observed (data not shown).

Three compounds (octanal, nonanal, and (*E*)-2-butenal) were produced in significantly highest amounts in the enzyme blank. Two of them (octanal and nonanal) were also produced in the two fatty acid blank samples (in a significantly lower level though), which could indicate autoxidation to a certain degree, but there was no increase during the 60 min of reaction. It is not obvious why these three compounds are produced in higher amounts in the enzyme blank than in the fatty acid samples with enzyme extract, unless a decomposition of the compounds was induced when the fatty acids were present. A decrease over time was observed for (*E*)-2-butenal when linoleic acid was added, but linolenic acid resulted in an increase over time.

Four alcohols, 1-propanol, 1-butanol, 1-pentanol, and 1-hexanol, were detected in the three samples with enzyme extract added and not in the fatty acid blank samples, which confirmed that activity of alcohol dehydrogenase was present in the crude leek extract (**Table 1**). Linoleic acid resulted in the highest concentration of 1-pentanol and 1-hexanol, whereas linolenic acid produced the highest amount of 1-butanol.

The model experiments were also performed with microwave heating of the solution to 90 °C (data not shown) instead of calcium chloride inactivation of the enzymes. This method of enzyme inactivation is relevant for the processing of food. The results showed the same trend as that for calcium chloride inactivation, but heat treatment gave higher concentrations of the compounds probably as a result of increased chemical reaction rate and autoxidation. Decanal, (*E*)-2-nonenal, (*E*,*E*)-2,4-hexadienal, (*E*,*E*)-2,4-nonadienal, and (*E*,*Z*)-2,4-decadienal were only formed by microwave heating.

LOX originating from leek showed 4 times higher catalytic activity toward linoleic acid than toward linolenic acid. On the basis of the aroma results it, was concluded that leek LOX is specific in the production of hydroperoxides, as no products after the 9-hydroperoxide were detected, and essentially only products after the 13-hydroperoxide were detected. pH-Optimum of leek LOX was found to be 4.5−5.5 against linoleic acid, which was lower than that reported for other plant lipoxygenases. The substrate specificity investigations showed that leek HPL is not specific toward one of the hydroperoxides. A pronounced difference in required reaction time to produce the detected aroma compounds was found, some compounds developed instantly, whereas others still developed after a reaction time of 60 min.

ABBREVIATIONS USED

LOX, lipoxygenase; HPL, hydroperoxide lyase; ADH, alcohol dehydrogenase; 9-HPODE, 9-(S)-hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid; 13-HPODE,13-(S)-hydroperoxy-(9Z,11*E*)-octadecadienoic acid

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Paper 6

Nielsen G. S.; Larsen L. M.; Poll L. Impact of blanching and packaging atmosphere on the formation of aroma compounds during long-term frozen storage of leek (*Allium ampeloprasum* Var. *Bulga*) slices.

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Impact of Blanching and Packaging Atmosphere on the Formation of Aroma Compounds during Long-Term Frozen Storage of Leek (*Allium ampeloprasum* Var. *Bulga)* Slices

Ghita Studsgaard Nielsen*[†], Lone Melchior Larsen[‡] & Leif Poll[†]

[†]Department of Dairy and Food Science and LMC, Centre of Advanced Food Studies, The Royal Veterinary and Agricultural University, Rolighedsvej 30, 1958 Frederiksberg C, Denmark, and [‡]Chemistry Department, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark

*To whom correspondence should be addressed. Tel.: +45-35283435. Fax: +45-35283265. E-mail gni@kvl.dk)

Running title: Aroma changes in leek slices during frozen storage

ABSTRACT

Content of aroma compounds and catalytic activity of lipoxygenase (LOX), alliinase, hydroperoxide lyase (HPL), and alcohol dehydrogenase (ADH) were analyzed in unblanched and blanched 15-mm leek slices packed in atmospheric air (21% O_2) or 100% nitrogen (0% O_2) three times during 12 months of frozen storage (12 M). Total amount of sulfur compounds and total amount of aldehydes were greatly influenced by storage time, atmosphere, and blanching (concentration of sulfur compounds in fresh unblanched (UNB) slices = 1.35 mg/L, fresh blanched (B) slices = 1.09 mg/L, UNB 21% O_2 12 M = 0.656 mg/L, UNB 0% O_2 12 M = 2.11 mg/L, B 21% O_2 12 M = 1.14 mg/L, B 0% O_2 12 M = 1.59 mg/L). B 0% O_2 was closest to the original ratio between sulfur compounds and aldehydes after 12 M. Activity of HPL and alliinase was totally lost after 12 M, ADH showed minimal activity, whereas LOX (UNB 0% O_2) showed ~ 25% of the original activity. LOX was the most and HPL the least heat labile enzyme investigated.

KEYWORDS

Leek; flavor; frozen storage; lipoxygenase; hydroperoxide lyase; alcohol dehydrogenase; alliinase; packaging atmosphere; blanching.

INTRODUCTION

The chemical composition of *Allium* plants is characterized by a high amount of organic bound sulfur compared to most other plants (*1*). This determines the aroma of freshly cut leek and other *Allium* species, which mainly consist of numerous sulfur-containing volatile compounds. These compounds originate from the alliinase (EC 4.4.1.4) -catalyzed decomposition of the odorless nonvolatile precursors *S*-alk(en)yl cysteine sulfoxides to sulfenic acids (*2,3*). Sulfenic acids are highly reactive (*4*) and will quickly combine to form thiosulfinates. Thiosulfinates are

responsible for the odor of freshly cut leeks (*4,5*), but as they are relatively unstable (*2,6*) they will rearrange to form polysulfides and thiosulfonates. Thiosulfonates are transformed to the corresponding monosulfide, and polysulfides can rearrange to mono- and trisulfides, so the final products of the reactions will be a mixture of mono- and polysulfides with all of the possible combinations of the *S*-alk(en)yl radicals of common occurrence. The existence of these radicals is dependent of the species, in leek the presence of methyl-, ethyl-, propyl-, 1-propenyl-, and 2-propenyl- radicals has been reported (*4,6,7*).

The lipoxygenase pathway is another contributor to the formation of aroma in fresh and especially in stored leek slices. Lipoxygenase (EC 1.13.11.12) (LOX) catalyzes the peroxidation of polyunsaturated fatty acids with a (*Z,Z*)-1,4-pentadiene moiety (*8,9*) under the consumption of dioxygen. The polyunsaturated fatty acids are present in cell membranes, which has to be liberated before initiation of the reactions (*10*). This happens during the cutting and cleaning process of the vegetable, or by natural loss of cell structure during senescence. Volatile aldehydes are produced by the action of hydroperoxide lyase (HPL) upon the formed hydroperoxides (*8,11*). All of these aldehydes, both saturated and nonsaturated, can be further metabolized by alcohol dehydrogenase (EC 1.1.1.1) (ADH) to the corresponding alcohols (*11*). These fatty acid-derived compounds can also generate by autoxidation during the storage period (*12*), and both pathways will result in accumulation of the compounds in the tissue and in the headspace of retail packages.

The formation of aldehydes and alcohols contribute to the aroma of fresh leeks when present in minor concentration but will probably appear as off-flavors when present in larger amounts (*13,14*). This is not very pronounced in the fresh leek because of the pungency of thiosulfinates and thiopropanal-*S*-oxide (*6*). However, previous investigations of unblanched frozen leek slices (*13,15,16*), have shown that the storage period has a great influence on the aroma profile of the leek slices as the concentration of sulfur compounds decreases and aldehydes (saturated and nonsaturated) increases effectively during the storage.

Production of frozen vegetable often includes a blanching step mainly to destroy the catalytic activity of enzymes and prevent off-flavor formation. In addition microorganisms are killed and air is expelled from the tissue, which can reduce autoxidation during storage. But usually loss of flavor, vitamins, and texture are consequences of the blanching process and the subsequently cooling (*17,18*) and especially for leek slices the loss of texture is detrimental to the quality.

A previous investigation (*15*) on nitrogen packaging of frozen unblanched leek slices has shown that after 1 year of frozen storage the development of off-flavor was reduced and the content of sulfur compounds was kept almost at the level of fresh leek slices, when the slices were stored in 100% nitrogen. Consequently both blanching and nitrogen packaging could retard oxidation during the frozen storage, and thus result in less accumulation of off flavor in the leek slices, but as the nitrogen packaging does not influence texture, it is valuable to compare the two methods.

The objective of the present study was to investigate the impact of blanching and packaging atmosphere on aroma formation and enzyme activity in frozen leek slices stored for 12 months.

MATERIALS AND METHODS

Plant Material. Leeks (*Allium ampeloprasum,* var. *Bulga*) were harvested fully matured at Funen, Denmark in October 2001 and used for the experiment immediately after.

Chemicals. The reference compounds (purity ≥ 97%)1-propanethiol, dimethyl disulfide, methyl propyl disulfide, dipropyl disulfide, dimethyl trisulfide, 2,5-dimethyl thiophene, propanal, butanal, pentanal, hexanal, heptanal, octanal, nonanal, decanal, (*E*)-2-butenal, (*E*)-2-pentenal, (*E*)-2-hexenal, (*E*)-2-heptenal, (*E*)-2-nonenal, 2-methyl-(*E*)-2-butenal, 2-methyl-(*E*)-2-pentenal, (*E*,*E*)-2,4-heptadienal, (*E*,*E*)-2,4-decadienal, 2-heptanone, 2-octanone, 3-octanone, 1-propanol, 1-pentanol, 1-hexanol, 1-octen-3-ol, propanoic acid, hexanoic acid, and 2-pentyl furan were bought commercially from Sigma-Aldrich, Copenhagen, Denmark. (*E*,*Z*)-2,4-Heptadienal was occurring as an impurity in (*E*,*E*)-2,4-heptadienal. NADH and lactate dehydrogenase (LDH) were bought commercially from Roche Diagnostics Scandinavia AB, Hvidovre, Denmark. All other chemicals, except for *S*-methyl-L-cysteine sulfoxide and 9-(*S*)-hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid, were of analytical grade and were bought commercially from Sigma-Aldrich.

Synthesis of *S*-Methyl-L-cysteine Sulfoxide. Modified after (*19*). Fifty milliliters of *S*-methyl-L-cysteine (0.6 M in deionized water) and 3.80 mL of hydrogen peroxide (30%) were mixed for 24 hours at 24°C with agitation (100 rpm). The generated cysteine sulfoxide was precipitated by 50 mL of cold (3°C) ethanol for 12 hours at 3°C. Separation of the precipitation product was done through a paper filter. The precipitate was dried and afterwards dissolved in 5 mL of 0.2 M phosphate buffer (pH 6.0) and kept at 3°C in the dark until analysis.

Synthesis of 9-(S)-Hydroperoxy-(10*E*,12*Z*)-octadecadienoic Acid (9-HPODE). Sodium linoleate (1.25 mL, 10mM), 4.75 mL of air saturated 50 mM potassium phosphate buffer (pH 7.0), and 1.50 mL of potato tuber lipoxygenase solution were mixed, then agitated and purged with oxygen for 30 min. The solution was checked for absorbance at 234 nm to determine the content of 9-HPODE. According to Galliard and Phillips (*20*), potato tuber LOX almost exclusively converts linoleic acid into 9-HPODE.

Preparation of Potato Tuber Lipoxygenase Solution for Synthesis. Grated potato tubers (50 g) and 50 mL of tap water were homogenized for 30 s in a Waring commercial blender and filtrated through a paper filter. A 2-mL aliquot of the liquid was applied to a DEAD-Cellulose C545 column (anion exchanger) (Sigma-Aldrich, Denmark) followed by 6.0 mL of 50 mM potassium phosphate buffer (pH 7.0). Two fractions of 4.0 mL were collected; both were checked for activity, and the second one was used as the lipoxygenase solution.

Sample Preparation. Unblanched samples: The white part of the leek stem was cut into 15-mm slices and frozen immediately after in a blast freezer at −20 °C for 10 min, so that the center temperature reaches −20 °C. Blanched samples: The white part of the leek stem was cut into 15-mm slices and immediately after portions of 500 g slices were immersed in a pot with lid containing 3 liter of boiling water for 225 s. After the blanching the leek slices were dripped off

for 15 s, cooled down in crushed ice for 15 min and frozen in a blast freezer at −20 °C for 10 min, so that the center temperature reaches −20 °C.

Both unblanched and blanched frozen leek slices were packed in airtight glass jars with atmospheric air or 100% nitrogen as headspace and kept at −20 °C in the dark until analysis. Fresh Pax^{TM} oxygen absorbers (Type R 50 cc) from Multisorb Technologies, Inc. Buffalo, NY were added to the glasses with 100% nitrogen.

Experimental Design. Two individual samples of unblanched and blanched slices were analyzed at harvest time and subsequently two individual samples of unblanched and blanched slices packed in atmospheric air and in 100% nitrogen were analyzed after 6 and 12 months of frozen storage. Each sample was analyzed for composition of atmosphere in the glass jars, catalytic activity of LOX, HPL, ADH, and alliinase, and content of aroma compounds in three replicates.

Gas Analysis. The O_2, CO_2, and N_2 concentrations in the glass jars were determined by a Gaspace 2 atmosphere analyzer from Systech Instruments Ltd, Oxon, UK.

Preparation of Enzyme Extract. Enzyme extract was made by mixing 100 g of frozen leek slices with 100 mL of potassium phosphate buffer (50 mM, pH 7.0 added 0.1% Triton X-100) for 120 s in a Waring commercial blender. The slurry was kept on ice with agitation for 30 min (100 rpm), afterwards filtered through a paper filter until 35.0 mL was collected, and kept on ice until time of analysis. The enzyme extract was desalted by size exclusion chromatography through a PD-10 column (Amersham Biosciences, Sweden) with Sephadex G-25 Medium prior to analysis of HPL, ADH and Alliinase.

Lipoxygenase Assay. LOX was assayed in a continuous assay by measuring consumption of initial dioxygen by an YSI 5100 dissolved oxygen meter (YSI Inc., Yellow Springs, OH) at 30 °C, using linoleic acid as the other substrate. Calibration was done at 30 °C by air-saturated phosphate buffer (0.2 M, pH 6.0) (21% dissolved dioxygen) followed by addition of sodium dithionite (0% dissolved oxygen). To determine lipoxygenase activity, 27.2 mL of air-saturated tempered phosphate buffer (0.2 M, pH = 6.0) and 3.9 mL of enzyme extract was mixed in a 33-mL conical flask, and the measuring was started. After 30 s the reaction was initiated by adding 1.9 mL of sodium linoleate solution (10 mM). Dioxygen was monitored over 20 min measuring dioxygen content every second.

Blank samples were run on buffer added substrate or enzyme extract.

Calculation of LOX Activity. LOX activity was calculated as katals per kg of protein, and katals were defined as moles of O_2 consumed per second. This was done under the assumption that air-saturated phosphate buffer (0.2 M, pH 6.0) at 30 °C with a salt strength of 12.40 g/L contains 7.11 mg O_2/L (*21*). To determine the activity the dioxygen level (mmol of O_2) was plotted against time and the steepest slope (mmol O_2/s) on the curve was found using continuous linear regression over 20 s at a time.

Hydroperoxide Lyase Assay. HPL was assayed in a continuous coupled assay modified after (*22*). ADH converts the products of HPL's reaction on hydroperoxides, aldehydes, under the oxidation of NADH, which can be monitored spectrophotometrically at 340 nm.

Phosphate buffer (1.8 mL, 0.2 M, pH = 6.0), 300 µL of NADH (2.2 mM in deionized water), 200 µL of ADH (150 units in 100 µL potassium phosphate buffer (50 mM, pH 7.0)) and 300 µL of enzyme extract were transferred to a thermostatic (30 °C) 1-cm cuvette with a magnetic stirrer, and the measuring was started. After 60 s 400 µL of 9-HPODE was added. The reaction was monitored over 600 s by measuring every second on a HP 8453A UV-Vis spectrophotometer.

Blank samples were run on buffer, NADH, and ADH added substrate or enzyme extract.

Calculation of HPL Activity. HPL activity was calculated as katals per kg of protein and katals were defined as moles of hydroperoxide consumed per second. To determine the activity, the absorbance was plotted against time and the steepest slope, (absorbance/s) on the curve was found using continuous linear regression over 5 s at a time. Absorbance was converted to moles of hydroperoxide by the assumption that each mole of hydroperoxide metabolized produced one mole of aldehyde. Aldehydes are converted by alcohol dehydrogenase by the consumption of NADH 1:1. ε of NADH at 340 nm is 6220 L/mol*cm (*22*).

Alcohol Dehydrogenase Assay. ADH was assayed in a continuous assay by following the oxidation of NADH spectrophotometrically at 340 nm when adding hexanal.

Borate buffer (1.4 mL, 0.05 M, pH = 9.0), 300 µL of NADH (2.2 mM in deionized water) and 1000 µL of enzyme extract were transferred to a thermostatic (30 °C) 1-cm cuvette with a magnetic stirrer and the measuring was started. After 60 s, 300 µL of hexanal (100 ppm in deionized water) was added. The reaction was monitored over 600 s by measuring every second on a HP 8453A UV-Vis spectrophotometer.

Blank samples were run on buffer and NADH added substrate or enzyme extract.

Calculation of ADH Activity. Alcohol dehydrogenase activity was calculated as katals per kg of protein and katals were defined as moles of hexanal consumed per second. Moles of hexanal were calculated on the basis of NADH under the assumption that hexanal and NADH were metabolized 1:1 and by the conditions described under calculation of HPL activity.

Alliinase Assay. Alliinase was assayed in a continuous coupled assay. LDH converts pyruvate, one of the products of alliinases reaction on *S*-methyl-L-cysteine sulfoxide, under the oxidation of NADH, which can be monitored spectrophotometrically at 340 nm.

Phosphate buffer (2.1 mL, 0.2 M, pH = 6.0), 300 µL of NADH (2.2 mM in deionized water), 10 µL of pyridoxal-5'-phosphate (6 mM in deionized water), 50 µL of LDH (900 units in 1 mL 50% glycerol (v/v), pH 6.5) and 600 µL of enzyme extract were transferred to a thermostatic (30 °C) 1-cm cuvette with a magnetic stirrer and the measuring was started. After 60 s 40 µL of *S*-methyl-L-cysteine sulfoxide (0.18 M in 0.2 M phosphate buffer, pH 6.0) was added. The reaction was monitored over 600 s by measuring every second on a HP 8453A UV-Vis spectrophotometer.

Blank samples were run on buffer, NADH, pyridoxal-5'-phosphate, and LDH added substrate or enzyme extract.

Calculation of Alliinase Activity. Alliinase activity was calculated as katals per kg of protein and katals were defined as moles of *S*-methyl-L-cysteine sulfoxide consumed per second. Moles of *S*-methyl-L-cysteine sulfoxide were calculated on the basis of NADH under the assumption that each mole of *S*-methyl-L-cysteine sulfoxide metabolized produced one mole of pyruvate.

Pyruvate is converted by lactate dehydrogenase by the consumption of NADH 1:1. Other conditions as described under calculation of HPL activity.

Determination of Protein Content. Protein content in the enzyme extraction was determined by Coomassie brilliant blue, according to the method described in US/EG Bulletin 1069 from Bio-Rad Life Science Group, Hercules, CA, using lyophilized bovine serum albumin as the standard protein.

Dynamic Headspace Analysis. Aroma compounds were isolated by dynamic headspace with nitrogen (purity = 99.8%) as purge gas. Fresh or frozen leek slices (100 g) were crushed with 150 mL of tap water and 4 mL of internal standard (50 ppm of 4-methyl-1-pentanol in tap water) for 120 s in a Waring commercial blender. After the mixture had been transferred to a 1-L glass flask by adding another 150 mL of tap water, it was left for 10 min at 30 °C with agitation (200 rpm) to equilibrate the temperature before purging. Aroma compounds were trapped on 250 mg of Tenax GR (mesh size = 60/80, Buchem bv, Apeldoorn, The Nederlands) for 45 min with a nitrogen flow of 75 mL/min and agitation (200 rpm).

Desorption of aroma compounds was done thermally by an ATD 400 automatic thermal desorption system (Perkin-Elmer, Bucks, England). Desorption temperature of the trap to the cold trap (contains 30 mg tenax GR, temperature 5 °C) was 250 °C for 15 min with a helium flow of 60 mL/min. Desorption temperature of the cold trap was 300 °C for 4 min, with a helium flow of 31 mL/min and an outlet split ratio of 1:30. Separation was performed by a GC-MS (HP G1800 A GCD system) with the following conditions: column, DB Wax from J&W Scientific, CA (30-m x 0.25-mm i.d. x 0.25-µm film thickness); carrier gas, helium; start flow, 1 mL/min; split ratio, none; column pressure, 48 kPa (constant); oven program, 45 °C for 10 min, 6 °C/min to 240 °C, constant at 240 °C for 30 min. The mass selective detector was operated in the electron ionization mode (ionization energy, 70 eV), and the *m*/*z* (mass/charge) ratio ranged between 10 and 425. Identification was done by probability-based matching with mass spectra in the G1035A Wiley library (Hewlett-Packard). Identity was confirmed by checking with mass spectra and retention indices obtained in the laboratory from reference compounds for the following compounds: 1-propanethiol, dimethyl disulfide, methyl propyl disulfide, dipropyl disulfide, dimethyl trisulfide, 2,5-dimethylthiophene, propanal, butanal, pentanal, hexanal, heptanal, octanal, nonanal, decanal, (*E*)-2-butenal, (*E*)-2-pentenal, (*E*)-2-hexenal, (*E*)-2-heptenal, (*E*)-2-nonenal, 2-methyl-(*E*)-2-butenal, 2-methyl-(*E*)-2-pentenal, (*E*,*E*)-2,4-heptadienal, (*E*,*E*)-2,4-decadienal, 2-heptanone, 2-octanone, 3-octanone, 1-propanol, 1-pentanol, 1-hexanol, 1-octen-3-ol, propanoic acid, hexanoic acid, and 2-pentyl furan.

Quantification. A 10-µL aliquot of each of the above listed reference compounds was dissolved in 20 mL of 96% ethanol and diluted with tap water into four appropriate concentrations depending on the concentration of the compounds in the leeks. Sucrose (1.3%), which equals the sugar content of leeks, was added to the final solutions. Dynamic headspace analysis was performed on 400 mL of the solutions added 4 mL of internal standard (50 ppm 4-methyl-1-pentanol in tap water) with the same flow, time, and temperature conditions as applied to the leek samples. Each reference compound was analyzed in triplicate in all four concentrations. The obtained peak area divided by the peak area of the internal standard was used to calculate the

concentration of the same compound in the leeks from the peak area of the compound divided by the peak area of the internal standard. Methyl 2-propenyl disulfide, methyl propenyl disulfide, and ethyl 1-methylethyl disulfide were quantified after the obtained peak area of methyl propyl disulfide, propyl 2-propenyl disulfide, propyl propenyl disulfide, propyl butyl disulfide, and propyl pentyl disulfide after dipropyl disulfide, diisopropyl trisulfide, and propyl propenyl trisulfide after dimethyl trisulfide, 3,4- or 2,4-dimethyl thiophene after 2,5-dimethyl thiophene, 2-ethyl-(*E*)-2-butenal after 2-methyl-(*E*)-2-pentenal, 3-octen-2-one and 3,5-octadiene-2-one after 3-octanone, and 2,5-dimethyl furan after 2-pentyl furan.

The retention time indices (RTI) of the volatile compounds were calculated with a mixture of hydrocarbons (C_9-C_{26}) as references, according to the method of (*23*).

Statistical Analysis. Analysis of variance was carried out on catalytic activity of LOX, HPL, ADH, and alliinase, and on each of the aroma compounds using the one-way and the factorial ANOVA procedure in the Analyst part of SAS, version 8.2, SAS institute Inc., Cary, NC. The effect of the storage period, blanching, and packaging atmosphere was tested. Sample means were compared by Duncan's multiple range test. A significance level of 5% was applied.

RESULTS AND DISCUSSION

The unblanched slices packed in atmospheric air (UNB 21% O_2) modified the atmosphere in the glass jars to 14.5% O_2 and 5.6% CO_2 after 12 months of frozen storage (**Table 1**). This indication of respiration in the leek slices during frozen storage was also found in a previous study (*15*), which also revealed that greater extent of shredding gave less modification of the atmosphere. The blanched slices packed in atmospheric air (B 21% O_2) did not modify the atmosphere. The nitrogen packaging, both unblanched (UNB 0% O_2) and blanched (B 0% O_2), kept the atmosphere in the jars constant at 98-99% N_2 except for the UNB 0% O_2 after 6 months of storage, which for some reason held 7.1% O_2, 1.0% CO_2 and 91.9% N_2. The atmosphere composition after 12 months showed that minor respiration also occurred in the UNB 0% O_2 as these samples contained 0.5% O_2 and 1.5% CO_2, whereas the B 0% O_2 held 1.1% O_2 and 0.0% CO_2.

UNB 21% O_2 samples produced a total amount of sulfur compounds of 1.35 mg/L as fresh which decreased significantly to 0.656 mg/L after 12 month of frozen storage (**Table 2**). This is consistent with previous reported results (*13,15*). Five of the sulfur compounds (ethyl 1-methylethyl disulfide, propyl butyl disulfide, diisopropyl trisulfide and both isomer of propyl propenyl trisulfide) actually increased significantly during storage and for at least three of them, this was substantial (**Table 3**). Five other compounds (methyl 2-propenyl disulfide, one isomer of propyl propenyl disulfide, propyl pentyl disulfide, and 2,5-dimethyl thiophene) were present in constant amounts during the storage period, whereas the amount of the remaining nine sulfur compounds was declining significantly through the period.

The blanching process reduced the total production of sulfur compounds in the fresh samples to 1.09 mg/L (**Table 2**) but still six compounds (ethyl 1-methylethyl disulfide, dipropyl disulfide,

one isomer of propyl propenyl disulfide, propyl butyl disulfide, propyl pentyl disulfide, and 3,4 or 2,4-dimethyl thiophene) were produced in statistically equal amounts and 1-propanethiol and 2,5-dimethyl thiophene were increased (**Table 3**). These two compounds have by other authors been shown to increase during heat treatment of onions (*1,24*). The blanching process stabilized the decomposition of sulfur compounds during frozen storage as the B 21% O_2 samples stored for 12 months contained statistically the same amount of sulfur compounds (1.14 mg/L) (**Table 2**) as the fresh blanched samples, and higher amounts than the UNB 21% O_2 12 M samples.

About half of the aldehydes were not detected at all in the fresh unblanched slices (**Table 3**), but they developed during frozen storage and the ones already present in the fresh unblanched slices also increased during storage. Exceptions were propanal, (*E*)-2-hexenal, (*E*,*Z*)-2,4-heptadienal, and (*E*,*E*)-2,4-heptadienal which were present in statistically equal amounts before and after frozen storage. The total amount of aldehydes (not including propanal, 2-methyl-(*E*)-2-butenal, and 2-methyl-(*E*)-2-pentenal, which are breakdown products from the lachrymatory factor thiopropanal-*S*-oxide (*25*)) increased from 0.681 mg/L to 7.86 mg/L (**Table 2**) in the UNB 21% O_2 12 M and the majority of this effectual increase was due to pentanal, hexanal, and (*E*)-2-butenal. Previous investigations (*13,15*) showed a equivalent increase of aldehydes. In the blanched samples this development was not as noticeable as the total amount of aldehydes was 0.477 mg/L in the fresh samples and 2.13 mg/L in the B 21% O_2 12 M samples, but still the difference was significant. Six compounds ((*E*)-2-butenal, (*E*)-2-nonenal, (*E*,*Z*)-2,4-heptadienal, (*E*,*E*)-2,4-heptadienal, (*E*,*E*)-2,4-decadienal, and 2-ethyl-(*E*)-2-butenal) did not develop at all in the B 21% O_2 during the 12 months of frozen storage and propanal decreased (**Table 3**).

There was a minor difference between the fresh unblanched and blanched samples as six of the aldehydes (butanal, hexanal, heptanal, (*E*)-2-hexenal, (*E*)-2-heptenal, and 2-methyl-(*E*)-2-pentenal) decreased after blanching and pentanal and decanal increased. After 12 months of frozen storage this difference was more pronounced as the total amount of aldehydes was 7.86 mg/L in the UNB 21% O_2 samples and 2.13 mg/L in the B21% O_2 samples (**Table 2**).

The alcohols, ketones, furans, and acids were not formed in the fresh samples but developed during frozen storage in the unblanched samples (**Table 3**). In the blanched samples this development was not seen, as many of the compounds were not formed at all. An exception was 1-octen-3-ol, which developed from 0.0 mg/L to 0.222 mg/L in the B21% O_2 12 M.

Whereas UNB 21% O_2 showed a decline of over 50% of the total content of sulfur compounds during frozen storage, the nitrogen packaging had a very positively effect on the keeping of sulfur compounds as the total content of sulfur compounds after 12 months of frozen storage under nitrogen was 2.11 mg/L compared to the 1.35 mg/L in the fresh slices (**Table 2**) and 12 of the compounds (methyl propyl disulfide, ethyl 1-methylethyl disulfide, dipropyl disulfide, one isomer of propyl propenyl disulfide, propyl 2-propenyl disulfide, propyl butyl disulfide, propyl pentyl disulfide, diisopropyl trisulfide, both isomers of propyl propenyl trisulfide, 1-propanethiol

and 2,5-dimethyl thiophene) actually increased significantly during storage (**Table 4**). This signifies that the UNB 0% O_2 samples stored for 12 months had the statistically highest total concentration of sulfur compounds of all. An increase of sulfur compounds altogether was also seen in the blanched samples stored under nitrogen, but 10 of the sulfur compounds (methyl propyl disulfide, methyl 2-propenyl disulfide, dipropyl disulfide, one isomer of propyl propenyl disulfide, propyl butyl disulfide, propyl pentyl disulfide, dimethyl trisulfide, diisopropyl trisulfide, one isomer of propyl propenyl trisulfide and 2,5-dimethyl thiophene) were present in statistically equal amounts before and after 12 months of frozen storage under nitrogen. The observed increase was mostly due to the development of 3,4- or 2,4-dimethyl thiophene, which increased almost 5 times during storage.

The development of aldehydes was also significantly influenced by storage under nitrogen as the total amount in the UNB 0% O_2 samples after frozen storage was 4.63 mg/L compared to 7.86 mg/L in UNB 21% O_2 (**Table 2**). When comparing the two blanched samples (B 21% O_2 and B 0% O_2) with UNB 0% O_2 (**Table 3-4**) after 12 months of frozen storage most of the aldehydes were present in statistically higher amounts in the UNB 0% O_2. The crucial difference between the three treatments though, was the development of hexanal which accounts for almost all of the difference in the total content of aldehydes. The formation of this compound should be inhibited by low oxygen, as it is a product of the lipoxygenase pathway and autoxidation, and this was also demonstrated by (*15*). Blanching prevented the formation of this compound, which indicated that the large accumulation was due to enzymatic activity rather than autoxidation. The production of (*E*,*E*)-2,4-decadienal indicated the same as this compound was only developed in the unblanched samples stored in both atmospheres. This was also the case with (*E*)-2-butenal, (*E*)-2-nonenal, 2-ethyl-(*E*)-2-butenal, (*E*,*Z*)-2,4-heptadienal and (*E*,*E*)-2,4-heptadienal.

Most of the ketones, alcohols, acids and furans were increased in the UNB 0% O_2 during storage, whereas most of these compounds were not detected at all in the B 0% O_2 samples after 12 M and only 3-octanone and 2-pentyl furan increased in the B 0% O_2.

Investigations of LOX activity (**Figure 1a**) showed that activity decreased significant in the two unblanched samples and activity was influenced by the packaging atmosphere as UNB 0% O_2 gave significant higher activity than UNB 21% O_2 both at 6 and 12 months of frozen storage. This stabilizing effect of nitrogen was also demonstrated by (*15*). Blanching time was chosen so that there was no lipoxygenase activity left right after blanching, and the blanched samples showed a constant low activity all through the frozen storage, which was not influenced by the packaging atmosphere. Unblanched leek slices lost activity during frozen storage but the UNB 0% O_2 still had 25% of the initial activity left after 12 months while the UNB 21% O_2 was statistically equal to the blanched samples.

Alliinase activity (**Figure 1b**) also demonstrated a decline in activity for the unblanched samples. In this case there was no difference between the two packaging atmospheres, which is probably a

consequence of oxygen not being involved in the alliinase-catalyzed reaction. The two blanched samples showed very low activity right after the blanching process, but still a significant but minor loss of activity of alliinase occurred during frozen storage. This demonstrated that alliinase was not totally inactivated by the blanching procedure, and that alliinase might be more heat resistant than LOX. After 6 months of frozen storage the activity of unblanched samples was still significantly higher than the blanched samples but at 12 months the activity was equal. This demonstrate lack of freezing stability of alliinase which is in agreement with (*26*), who reported denaturation of alliinase during freezing. Freeman & Whenham (*27*) found intact activity of alliinase during 30 days of frozen storage of whole onion bulbs, and Wäfler et al. (*28*) found that the freezing stability of alliinase is depending on the freezing technique more than the actual frozen storage, and only slow freezing led to loss of alliinase activity due to destruction of cell structure. Our results indicate that the storage time also influences the activity of alliinase.

Investigations of HPL activity (**Figure 1c**) evinced that HPL was not properly inactivated by the blanching process as the blanched fresh samples showed statistically the same activity as the unblanched fresh samples. All samples declined significantly during the frozen storage with no consequence of storage atmosphere. After 6 month of frozen storage the activity had dropped to less than half of the initial activity and after 12 month of frozen storage practically no activity was detected. This demonstrated that activity of HPL was sensible to frozen storage over longer periods.

All treatments had a restrictive influence on ADH activity during the 12 M of frozen storage (**Figure 1d**). UNB 21% O_2 showed statistically the same activity after 6 months though, but declined in the last 6 months of the storage period, whereas UNB 0% O_2 dropped significant during the whole period. After 12 months of frozen storage the two unblanched samples were equal but statistically higher than the two blanched samples. The blanched samples did show minor activity in the fresh samples indicating that the blanching process was not sufficient to inactivate ADH. This activity remained almost constant during the storage period with a diminutive but significant decrease after 12 M. B 0% O_2 showed a minor increase after 6 months of frozen storage, which decline again.

In this study none of the four enzymes investigated was stable during frozen storage for longer periods, but whereas alliinase and HPL totally lost activity, LOX and ADH were still able to show some activity after 12 month of frozen storage in the unblanched samples. In the case of LOX this was influenced by the storage atmosphere as 0% O_2 gave highest activity. LOX was on the other hand the most heat labile enzyme of the four investigated. LOX is the initiator of the lipoxygenase pathway and that combined with the deterioration of texture with prolonged blanching time probably makes it sensible to choose the blanching time on the basis of LOX, which was also suggested by (*18,29*). While the reactions of LOX, HPL and ADH mostly are unwanted, the action of alliinase during frozen storage would probably influence positively on the headspace in retail packages of frozen leek slices.

The aroma analyses showed that storage in 100% nitrogen without blanching actually enhanced the content of sulfur compounds in the leek slices tremendous when compared to the fresh samples, but it also enhanced the production of aldehydes. In the case of aldehydes this could be a result of the better keeping of LOX activity found in the UNB 0% O_2, which will result in formation of aldehydes during the crushing of leek slices prior to headspace analysis. This reflects the situation of thawing of the leek slices during cooking, and this activity is of course dependent on the condition of the enzymes at the actual time. In the case of sulfur compounds, alliinase activity should not be affected by the atmosphere, but nitrogen might have a preservative effect on the sulfur compounds or the precursors.

Comparison of the blanched and unblanched slices showed that the development of aldehydes was both a result of autoxidation and enzymatic activity. When stored in 21% O_2 the unblanched samples produced almost four times more aldehydes that the blanched samples, which indicate that mainly enzymatic activity is responsible for this production. This difference is both due to accumulation during the frozen storage and due to activity during the crushing of leek slices as explained above. The results show that it is possible to restrain autoxidation by packing without oxygen as the B 0% O_2 after 12 M had the same content of aldehydes as the fresh unblanched and blanched samples whereas the concentration in B 21% O_2 after 12 M had tripled. When comparing the ratio of total amount of sulfur compounds to total amount of aldehydes after 12 months of frozen storage the blanched samples stored in 100% nitrogen came closest to the ratio found in the fresh unblanched leek slices.

ABBREVIATIONS USED

UNB 21% O_2, unblanched slices packed in atmospheric air; B 21% O_2, blanched slices packed in atmospheric air; UNB 0% O_2, unblanched slices packed in 100% nitrogen; B 0% O_2, blanched slices packed in 100% nitrogen; 0 M, fresh leek; 12 M, 12 months of frozen storage; LOX, lipoxygenase; HPL, hydroperoxide lyase; ADH, alcohol dehydrogenase; LDH, lactate dehydrogenase; 9-HPODE, 9-(S)-hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid

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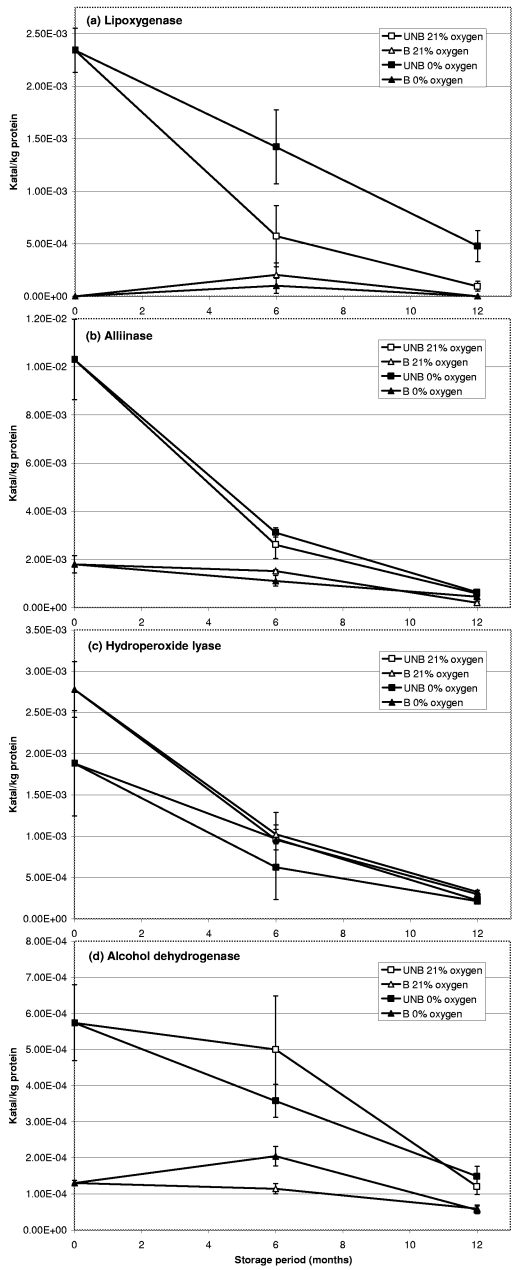

Figure 1. Specific activity of lipoxygenase (a), alliinase (b), hydroperoxide lyase (c), and alcohol dehydrogenase (d) analyzed in leek slices during frozen storage. UNB = unblanched. B = blanched. Packaging atmosphere is indicated as: 21% oxygen = atmospheric air, 0% oxygen = 100% nitrogen. Vertical bars indicate standard deviation.

Table 1. Atmosphere Composition in % in the Glass Jars during Storage of Frozen Leek Slices

storage period (month)		6	12
UNB[a] 21% O_2[b]	O_2	17.7	14.5
	CO_2	3.5	5.6
	N_2	78.8	79.9
B[c] 21% O_2	O_2	20.6	20.0
	CO_2	0.3	0.6
	N_2	79.1	79.4
UNB 0% O_2[d]	O_2	7.1	0.5
	CO_2	1.0	1.5
	N_2	91.9	98.0
B 0% O_2	O_2	2.1	1.1
	CO_2	0.0	0.0
	N_2	97.9	98.9

[a]Unblanched slices. [b]Initial atmosphere: atmospheric air. [c]Blanched slices. [d]Initial atmosphere: 100% nitrogen.

Table 2. Total[a] of Sulfur Compounds and Aldehydes Found in Fresh Leek (0 M) and in Leek Stored Frozen for 12 Months (12 M)

Compound	Sulfur	Aldehydes[b]
UNB[c] 0 M	1.35±0.451	0.681±0.206
UNB 21% O_2[d] 12 M	0.656±0.181	7.86±2.25
UNB 0% O_2[e] 12 M	2.11±0.216	4.63±0.774
B[f] 0 M	1.09±0.228	0.477±0.105
B 21% O_2 12 M	1.14±0.090	2.13±0.404
B 0% O_2 12 M	1.59±0.129	0.647±0.042

[a]All values are in mg/L±standard deviation. [b]Not including propanal, 2-methyl-2-butenal, and 2-methyl-2-pentenal, see text. [c]Unblanched slices. [d]Atmospheric air. [e]100% nitrogen. [f]Blanched slices.

Table 3. Aroma Compounds[a] Found in Fresh Leek (0 M) and in Leek Stored Frozen for 12 Months (12 M) in Atmospheric Air

compound	RTI[b]	UNB[c] 21% O_2 0 M	UNB 21% O_2 12 M	B[d] 21% O_2 0 M	B 21% O_2 12 M
sulfur compounds					
1-propanethiol	857	0.0432±0.0174	0.0269±0.00404	0.281±0.0672	0.0514±0.00887
dimethyl disulfide	1086	0.0363±0.0166	0.00355±0.000784	0.00706±0.00215	0.00714±0.000547
methyl propyl disulfide	1242	0.0917±0.0466	0.0636±0.0258	0.0168±0.00658	0.0247±0.00248
methyl 2-propenyl disulfide[e]	1296	0.000123±0.0000728	0.0000599±0.0000132	0.0000284±0.0000354	0.00±0
methyl propenyl disulfide[e,f]	1263	0.0508±0.0166	0.00266±0.000642	0.0318±0.00596	0.0339±0.00410
methyl propenyl disulfide[e,f]	1292	0.166±0.0586	0.00884±0.00304	0.0570±0.0120	0.0899±0.00905
ethyl 1-methylethyl disulfide[e]	1319	0.000314±0.000170	0.00227±0.000718	0.000166±0.000101	0.00±0
dipropyl disulfide	1387	0.62±0.180	0.387±0.107	0.461±0.218	0.500±0.0609
propyl 2-propenyl disulfide[e]	1436	0.0000660±0.0000404	0.0000739±0.0000256	0.00±0	0.0000490±0.0000117
propyl propenyl disulfide[e,f]	1422	0.00754±0.00271	0.00633±0.00231	0.00856±0.00146	0.00940±0.00103
propyl propenyl disulfide[e,f]	1438	0.0858±0.0378	0.0271±0.0123	0.0306±0.00424	0.0435±0.00390
propyl butyl disulfide[e]	1493	0.00±0	0.0000117±0.00000256	0.00±0	0.00±0
propyl pentyl disulfide[e]	1568	0.00±0	0.0000395±0.0000446	0.00±0	0.0000115±0.00000205
dimethyl trisulfide	1376	0.0383±0.0157	0.00240±0.000566	0.00182±0.000525	0.00240±0.000566
diisopropyl trisulfide[e]	1656	0.0212±0.00540	0.0695±0.0230	0.00888±0.00409	0.0151±0.00192
propyl propenyl trisulfide[e,f]	1781	0.000984±0.000161	0.00161±0.000602	0.000328±0.0000729	0.000486±0.000113
propyl propenyl trisulfide[e,f]	1770	0.00120±0.000188	0.00180±0.000619	0.000370±0.0000974	0.000583±0.000141
2,5-dimethyl thiophene	1190	0.00498±0.000679	0.00464±0.00440	0.0109±0.00238	0.0100±0.00125
3,4- or 2,4-dimethyl thiophene[e]	1253	0.185±0.0706	0.0501±0.0121	0.172±0.0270	0.348±0.0352

aldehydes					
propanal	762	2.86±0.336	2.16±0.712	2.42±1.66	0.705±0.112
butanal	867	0.00612±0.000828	0.0804±0.00601	0.00±0	0.0245±0.00155
pentanal	984	0.0468±0.0121	1.55±0.212	0.375±0.102	0.799±0.0514
hexanal	1111	0.313±0.0878	4.74±1.42	0.0267±0.00320	0.784±0.154
heptanal	1197	0.0262±0.00323	0.125±0.0322	0.0207±0.00216	0.0753±0.00964
octanal	1299	0.00±0	0.0132±0.00315	0.00±0	0.0161±0.00338
nonanal	1398	0.00±0	0.0114±0.00503	0.00±0	0.0289±0.00361
decanal	1510	0.00±0	0.000885±0.000474	0.000847±0.000358	0.00163±0.000388
(*E*)-2-butenal	1046	0.00±0	0.652±0.0519	0.00±0	0.00±0
(*E*)-2-pentenal	1147	0.00±0	0.109±0.0597	0.00±0	0.0297±0.0103
(*E*)-2-hexenal	1230	0.167±0.108	0.0862±0.0170	0.00728±0.00516	0.0130±0.00277
(*E*)-2-heptenal	1334	0.0545±0.00364	0.140±0.0835	0.0464±0.00275	0.357±0.194
(*E*)-2-nonenal	1532	0.00±0	0.00317±0.00210	0.00±0	0.00±0
2-methyl-(*E*)-2-butenal	1113	0.00±0	3.01±0.257	0.00±0	0.0900±0.00600
2-ethyl-(*E*)-2-butenal[e]	1166	0.00±0	0.0825±0.0100	0.00±0	0.00±0
2-methyl-(*E*)-2-pentenal	1176	2.55±0.285	0.101±0.0252	0.0289±0.00275	0.210±0.0325
(*E*,*Z*)-2,4-heptadienal	1464	0.0414±0.0109	0.0223±0.0197	0.00±0	0.00±0
(*E*,*E*)-2,4-heptadienal	1490	0.0248±0.00193	0.0148±0.0136	0.00±0	0.00±0
(*E*,*E*)-2,4-decadienal	1789	0.00±0	0.226±0.485	0.00±0	0.00±0

ketones					
2-heptanone	1180	0.00±0	0.00681±0.000633	0.00±0	0.00±0
2-octanone	1297	0.00±0	0.0199±0.00176	0.00±0	0.00±0
3-octanone	1272	0.00±0	0.0139±0.00194	0.00±0	0.00±0
3-octen-2-one[e]	1345	0.00±0	0.00675±0.000420	0.00±0	0.00±0
3,5-octadiene-2-one[e]	1516	0.00±0	0.00604±0.00180	0.00±0	0.00±0
alcohols					
1-propanol	1049	0.563±0.163	0.00±0	0.324±0.169	0.483±0.0409
1-pentanol	1274	0.00±0	0.471±0.135	0.00±0	0.00±0
1-hexanol	1371	0.00±0	0.153±0.0361	0.00±0	0.00±0
1-octen-3-ol	1458	0.00±0	0.0642±0.0132	0.00±0	0.222±0.107
acids					
propanoic acid	1564	0.0406±0.0143	0.123±0.0231	0.0286±0.00737	0.0161±0.00407
hexanoic acid	1816	0.0591±0.0302	1.89±1.01	0.0289±0.00738	0.0633±0.0242
furans					
2,5-dimethyl furan[e]	958	0.00498±0.000679	0.0228±0.00647	0.00±0	0.00±0
2-pentyl furan	1249	0.0157±0.00473	0.0335±0.0327	0.00445±0.000495	0.0190±0.00366

[a]All values except RTI are in mg/L ± standard deviation. [b]Retention time index. [c]Unblanched slices. [d]Blanched slices. [e]Compound was identified by GC-MS alone and quantified by peak area of another compound, see Materials and Methods for details. [f](*Z*) and (*E*) isomers.

Table 4. Aroma Compounds[a] Found in Leek Stored Frozen for 12 Months (12 M) in 100% Nitrogen

compound	RTI[b]	UNB[c] 0% O_2 12 M	B[d] 0% O_2 12 M
sulfur compounds			
1-propanethiol	857	0.0782±0.0115	0.0964±0.0380
dimethyl disulfide	1086	0.00349±0.00128	0.00131±0.000174
methyl propyl disulfide	1242	0.161±0.0251	0.0161±0.00309
methyl 2-propenyl disulfide[e]	1296	0.0000942±0.0000154	0.00±0
methyl propenyl disulfide[e,f]	1263	0.00975±0.0154	0.0249±0.00219
methyl propenyl disulfide[e,f]	1292	0.0285±0.00503	0.0806±0.00225
ethyl 1-methylethyl disulfide[e]	1319	0.00655±0.000979	0.00±0
dipropyl disulfide	1387	1.03±0.131	0.517±0.0859
propyl 2-propenyl disulfide[e]	1436	0.000196±0.0000491	0.0000399±0.00000998
propyl propenyl disulfide[e,f]	1422	0.0163±0.00305	0.00946±0.000891
propyl propenyl disulfide[e,f]	1438	0.0477±0.00899	0.0450±0.00235
propyl butyl disulfide[e]	1493	0.0000271±0.00000246	0.00±0
propyl pentyl disulfide[e]	1568	0.000119±0.0000426	0.00±0
dimethyl trisulfide	1376	0.00943±0.00199	0.00170±0.000262
diisopropyl trisulfide[e]	1656	0.622±0.0444	0.0123±0.00428
propyl propenyl trisulfide[e,f]	1781	0.00675±0.00178	0.000718±0.0000755
propyl propenyl trisulfide[e,f]	1770	0.00737±0.00196	0.000931±0.000122
2,5-dimethyl thiophene	1190	0.00976±0.00270	0.00924±0.000835
3,4- or 2,4-dimethyl thiophene[e]	1253	0.0790±0.00986	0.770±0.0567

aldehydes			
propanal	762	3.74±0.775	0.405±0.127
butanal	867	0.0287±0.00303	0.00535±0.00119
pentanal	984	0.575±0.0572	0.394±0.0333
hexanal	1111	2.95±0.654	0.107±0.0164
heptanal	1197	0.0724±0.0138	0.0353±0.00313
octanal	1299	0.00400±0.000903	0.00679±0.000688
nonanal	1398	0.0133±0.00175	0.0358±0.00646
decanal	1510	0.00135±0.000703	0.00±0
(*E*)-2-butenal	1046	0.166±0.0192	0.00±0
(*E*)-2-pentenal	1147	0.214±0.0284	0.00±0
(*E*)-2-hexenal	1230	0.234±0.0298	0.00976±0.00743
(*E*)-2-heptenal	1334	0.151±0.0243	0.0532±0.00551
(*E*)-2-nonenal	1532	0.00253±0.00198	0.00±0
2-methyl-(*E*)-2-butenal	1113	2.71±0.629	0.0331±0.00558
2-ethyl-(*E*)-2-butenal[e]	1166	0.0609±0.0182	0.00±0
2-methyl-(*E*)-2-pentenal	1176	0.358±0.0485	0.146±0.0222
(*E*,*Z*)-2,4-heptadienal	1464	0.0615±0.0113	0.00±0
(*E*,*E*)-2,4-heptadienal	1490	0.0392±0.00895	0.00±0
(*E*,*E*)-2,4-decadienal	1789	0.0493±0.0348	0.00±0

ketones			
2-heptanone	1180	0.000137±0.000191	0.00±0
2-octanone	1297	0.0000723±0.0000457	0.00±0
3-octanone	1272	0.00285±0.000308	0.0000417±0.0000365
3-octen-2-one[e]	1345	0.000358±0.0000717	0.00±0
3,5-octadiene-2-one[e]	1516	0.000850±0.000351	0.00±0
alcohols			
1-propanol	1049	5.04±0.789	0.00±0
1-pentanol	1274	0.390±0.0582	0.00±0
1-hexanol	1371	0.0312±0.00264	0.00±0
1-octen-3-ol	1458	0.0160±0.00812	0.00±0
acids			
propanoic acid	1564	0.296±0.0629	0.0163±0.0152
hexanoic acid	1816	1.656±0.361	0.00±0
furans			
2,5-dimethyl furan[e]	958	0.0000759±0.0000512	0.00±0
2-pentyl furan	1249	0.0472±0.0137	0.0165±0.00216

[a]All values except RTI are in mg/L ± standard deviation. [b]Retention time index. [c]Unblanched slices. [d]Blanched slices. [e]Compound was identified by GC-MS alone and quantified by peak area of another compound, see Materials and Methods for details. [f](*Z*) and (*E*) isomers.

Paper 7

Nielsen G. S.; Poll L. Influence of slice thickness and blanching on formation of aroma compounds during frozen storage of leek (*Allium ampeloprasum* Var. *Bulga*) slices.

This publication is in preparation for the 7th Wartburg symposium on flavor chemistry & biology, april **2004**. The work will be presented as a poster and a manuscript in the proceedings.

INFLUENCE OF SLICE THICKNESS AND BLANCHING ON FORMATION OF AROMA COMPOUNDS DURING FROZEN STORAGE OF LEEK (*ALLIUM AMPELOPRASUM* VAR. *BULGA*) SLICES

GHITA STUDSGAARD NIELSEN and Leif Poll

Department of Dairy and Food Science and LMC, Centre of Advanced Food Studies, The Royal Veterinary and Agricultural University, Rolighedsvej 30, 1958 Frederiksberg C, Denmark

The formation of aroma compounds in leek slices is mainly originating from two enzyme pathways. Alliinase activity results in formation of numerous sulphur compounds originating from cysteine. These compounds are characteristic for the aroma profile of freshly cut leeks. The lipoxygenase-catalyzed oxidation of polyunsaturated fatty acids leads to formation of compounds that will appear as off-flavour, when present in larger amounts.
Manufacturing of frozen leek slices does not always include a blanching step, mainly because this has an undesirable effect on the texture. Consequently, enzymes are still present during frozen storage, and enzymatic activity is possible both during storage and thawing. Previous studies have shown that the aroma profile of leek slices alters during frozen storage both due to enzymatic activity and autoxidation [1]. Blanching on the other hand also alters the aroma profile of leek as some aroma compounds decrease and some increase during the blanching process [2]. The present study investigates the impact of water blanching and slice thickness on the aroma profile of leek slices during frozen storage.
Water blanched leek slices and unblanched leek slices (4 mm and 15 mm) were frozen and stored for 12 month. The aroma compounds were analyzed at the start and after 6 and 12 months of frozen storage by dynamic headspace and thermal desorption. The results showed that blanching has an effectual impact on the concentration of sulphur compounds and aldehydes in the fresh leek slices independent of slice thickness, as the blanching procedure reduces the concentration of these aroma compounds. In the unblanched samples the overall concentration of aroma compounds is larger in the 4 mm compared to the 15 mm. After a frozen storage the blanched samples remains at the same low level, whereas sulphur compounds decrease in the unblanched samples and aldehydes (off-flavour) increase. 4 mm slices produce the largest increase of aldehydes probably due to larger percent of ruined cells per weight unit.

1 Nielsen GS, Larsen LM, Poll L (2003) Formation of aroma compounds and lipoxygenase (EC 1.13.11.12) activity in unblanched leek (*Allium ampeloprasum* Var. *Bulga*) slices during long-term frozen storage. J Agric Food Chem 51:1970-1976

2 Nielsen GS, Poll L (2003) Impact of water blanching on the retention and formation of aroma compounds in leeks (*Allium ampeloprasum* Var. *Lancelot*) In: Flavour research at the dawn of the twenty-first century. Proceedings of the Tenth Weurman Flavour Research Symposium. Eds: Le Quéré JL, Etiévant PX. Lavoisier, Cachan, France, p 678-681.

Table 1. Impact of slice thickness on production of volatiles in unblanched (UNB) and blanched (B) fresh leek slices (0 M) and in unblanched and blanched leek slices stored frozen at -20°C for 12 months (12 M).

		4 mm 0 M	**15 mm 0 M**	**4 mm 12 M**	**15 mm 12 M**
Sulfur[a]	UNB	4.90±0.46	1.35±0.45	2.41±0.69	0.656±0.18
	B	2.50±0.24	1.09±0.23	2.19±0.13	1.14±0.090
Aldehydes[b]	UNB	0.694±0.11	0.681±0.21	11.0±0.87	7.86±2.3
	B	0.498±0.18	0.477±0.11	1.67±0.35	2.13±0.40

[a] Total amount of sulfur compounds in mg/L. [b] Total amount of aldehydes except for propanal, 2-methyl-2-butenal and 2-methyl-2-pentenal in mg/L.

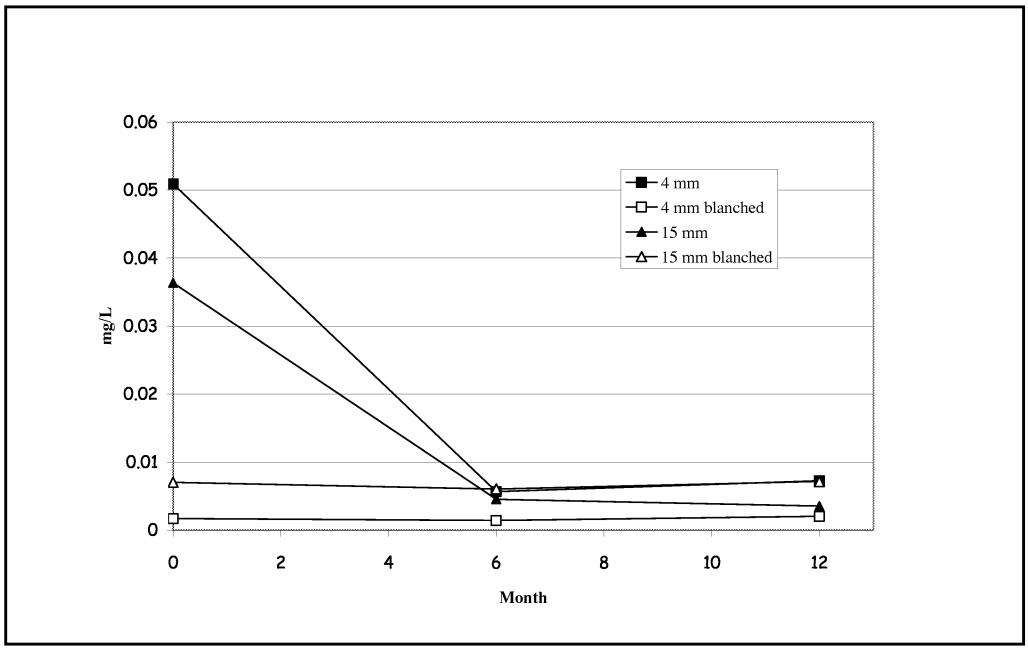

Figure 1. Development of dimethyl disulfide in leek slices during frozen storage.

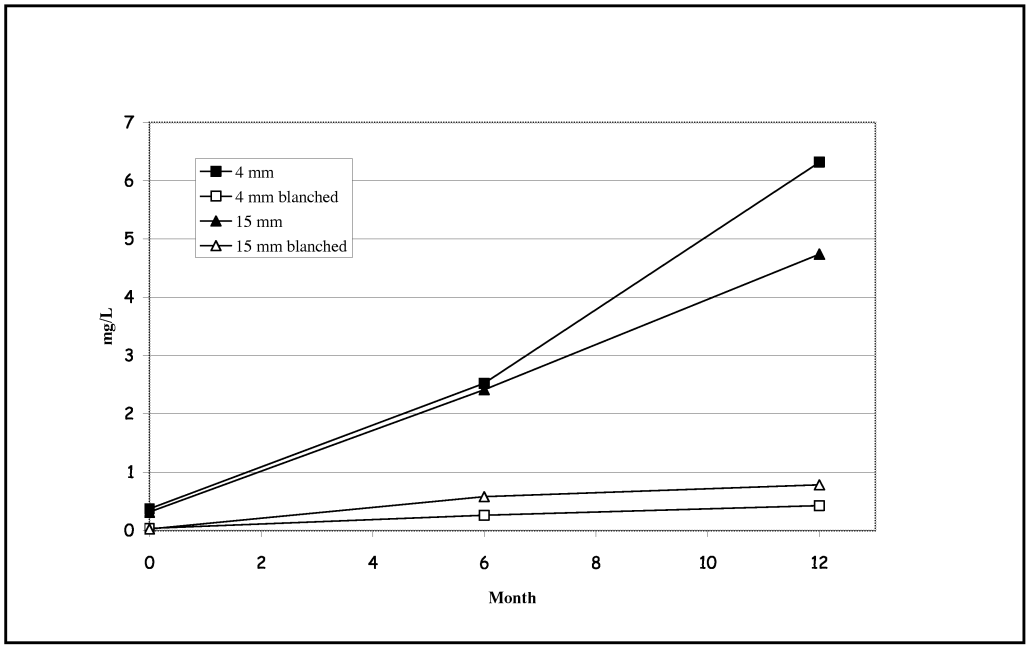

Figure 2. Development of hexanal in leek slices during frozen storage

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