

# **INVESTIGATION INTO THE SEMIOCHEMICAL COMMUNICATION INVOLVED IN NEONATAL OFFSPRING RECOGNITION IN SHEEP**

MARLIZE ZARETHA VIVIERS

Dissertation presented in fulfilment of the requirements for the degree

**Doctor of Philosophy in Chemistry**



at the

**University of Stellenbosch**

South Africa

Supervisor: Prof. B.V. Burger

Stellenbosch

Co-supervisor: Dr. M. le Roux

March 2010

*The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.*

## DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

March 2010



## ABSTRACT

Sheep husbandry substantially contributes to South Africa's economy. Lamb mortality is regarded as a major constraint to sustainable sheep production, and an important factor in lamb survival is the strong ewe–lamb bonding. This is a well-known phenomenon in all sheep races and strong evidence exists that the selective ewe–lamb bond formation has a pheromonal basis, in that the ewe can distinguish her lamb by its unique, familiar smell. Sheep farmers and scientists have examined various methods of making alien lambs acceptable to foster ewes. The survival rate of offspring could be substantially increased if a reliable method could be devised to prevent ewes from rejecting their own lambs or if ewes could be induced to accept alien lambs. A better understanding of the semiochemicals involved in this communication between ewe and offspring could facilitate the development of a method by which ewes can be coaxed into adopting foster lambs. This would positively contribute to efficient sheep production in South Africa.

The objective of this study was to characterise the volatile constituents present in the wool and the amniotic fluid collected from the Döhne Merino lambs to achieve a better understanding of the ewe–lamb semiochemical communication. The volatile organic constituents associated with the wool and amniotic fluid were characterised and a total of 133 volatile organic compounds were identified. Several compound classes are represented in these samples and the majority of these compounds have previously been identified in the excretions and secretions of numerous mammalian species.

A number of different aspects of ewe–lamb kin recognition were investigated. First, the odour profiles of twin lambs were compared to those of other twins. The results of these analyses revealed that the wool volatiles of twin lambs are qualitatively as well as quantitatively practically identical, but different from those of other lambs in the flock. Second, the change in the odour profiles of lambs was studied over a seven-day period. It was found that the odour profiles of lambs are not fixed, and for as long as a ewe makes use of olfactory recognition as the main mode of identification of her lamb she has to continue learning the odour of her lamb. Third, the amniotic fluid was investigated as a possible source of maternal labels, *i.e.* as chemical codes consisting of mixtures of

compounds in specific quantitative ratios that are imprinted on lambs by their mothers at birth. It was found that the amniotic fluids of first- and second-born lambs are qualitatively and quantitatively different. It is possible that the duration of the lambing birth process could contribute to this change in amniotic fluid composition. Fourth, the role of residual proteins in the dissemination of the semiochemicals involved in ewe-lamb communication was investigated and six proteins were identified as amniotic fluid and wool-associated proteins.

Bioassays carried out during the lambing season of 2009 confirmed the role of the odour of the lamb in ewe-lamb recognition, but bioassays carried out with synthetic pheromone formulations gave inconclusive results.

## OPSOMMING

Skaapboerdery lewer 'n aansienlike bydrae tot die ekonomie van Suid Afrika, maar lamsterftes is een van die kwellende probleme waarmee kleinveeboere te kampe het. Die eksklusiewe band wat binne die eerste paar uur na 'n lam se geboorte tussen die ooi en haar lam ontwikkel, is die hooftfaktor waarvan lammers se oorlewing afhang. Hierdie sterk binding tussen lam en ooi is 'n bekende verskynsel in alle skaap-rasse en dit is bekend dat 'n feromoon wat deur die lam vrygestel word, dit vir die ooi moontlik maak om haar lam aan sy unieke reuk uit te ken. Lamsterftes sou aansienlik verminder kon word as hierdie feromoon geïdentifiseer en gebruik kan word om ooie te verhoed om hulle eie lammers te verwerp en om die aanvaarding van vreemde lammers deur ooie moontlik te maak.

Die doel van hierdie studie was die chemiese karakterisering van die vlugtige organiese verbindings aanwesig in die amniotiese vloeistof en wol van Döhne Merino lammers ten einde 'n beter begrip van die semiochemiese kommunikasie tussen ooie en lammers te verkry. In die amniotiese vloeistof is 70 vlugtige organiese verbindings geïdentifiseer. Dieselfde verbindings asook 'n verdere 63 verbindings is in lammerwol geïdentifiseer. Die meerderheid van hierdie verbindings is reeds voorheen in verskeie soogdierafskeidings gevind.

Die studie het verskeie interessante aspekte van hierdie semiochemiese kommunikasie aan die lig gebring. Eerstens is daar gevind dat die samestelling van die vlugtige organiese materiaal in die wol van tweelinglammers, oftewel hul reukprofiel, feitlik identies is. Verder verskil die reukprofiel van 'n tweeling van dié van ander tweeling en enkelinge in die trop. Tweedens is gevind dat die reukprofiel van 'n lam nie by geboorte vasgelê word en so behoue bly nie, maar dat dit geleidelik verander oor ten minste die periode van sewe dae waaroor hierdie verskynsel bestudeer is. Dit noodsaak die ooi om haar geheue met betrekking tot die lam se reuk voortdurend te verfris gedurende die tydperk wat sy hoofsaaklik van haar reuksin gebruik maak om haar lam te herken. Derdens is die amniotiese vloeistof ondersoek as moontlike bron van 'n sogenaamde moedermerk, waardeur 'n chemiese mengsel van verbindings wat in 'n spesifieke kwantitatiewe samestelling by geboorte deur die ooi op haar lam agter gelaat

word. Ten opsigte van kwalitatiewe en kwantitatiewe samestelling, verskil die vlugtige organiese fraksie van die amniotiese vloeistof van die tweelinglam wat eerste gebore word van die amniotiese vloeistof van die lam wat tweede gebore word. Dit is onwaarskynlik dat amniotiese vloeistowwe wat ten opsigte van hul vlugtige organiese fraksies van mekaar verskil, nagenoeg identiese moedermerke op tweeling lammers sou kon laat. Vierdens is vasgestel dat spore van ses proteïene uit die amniotiese vloeistof op die wol van lammers agtergelaat word. Daar kon ongelukkig nie vasgestel word of hierdie proteïene 'n rol in die vrystelling van semiochemiese inligting speel nie.

Gedragstoetse wat tydens die lamseisoen van 2009 uitgevoer is, het bevestig dat die lam se reukprofiel 'n deurslaggewende rol in ooi-lam herkenning speel, maar gedragstoetse wat met sintetiese feromoonmengsels uitgevoer is, het onbesliste resultate opgelewer.

## ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to the following people or entities for their contributions throughout this study:

To my supervisor and mentor, Prof Ben Burger, thank you for your guidance, patience, inspiration and encouragement. Your input in both my life and this project will never be forgotten. I feel privileged to have been your student.

Dr Le Roux, thank you for your friendship, encouragement, and guidance. Thank you for providing expert advice and support and for proofreading the manuscript.

A big thank you to John Morris, Frankie Williams and Willem Daniels, who went to great lengths to help me during my sample collections and bioassays.

Thank you to Jens Kossmann and the Institute for Plant Biotechnology for making their labs available for the protein identification and characterisation part of this study.

To Prof N. J. L. Le Roux, thank you for your competent and enthusiastic assistance with statistical analyses.

Thank to Dr. Marietjie Stander and Fletcher Hiten of the Central Analytical Facility of Stellenbosch University for GC-HRMS analyses.

Thank you to Dr. Margie Hurndall for editing the manuscript.

Financial support is gratefully acknowledged from the National Research Foundation (NRF), the Ernst and Ethel Eriksen Trust, the Harry Crossley Trust, Stellenbosch University and LECUS.

All my friends at the Chemistry Department, especially to Christel Cronje for continuous encouragement, friendship and support. A special thank you to Jan Bekker for all your support and encouragement.

My sincerest gratitude to my parents, Chev and Amanda Viviers, for laying the foundation for the education that I now possess. Thank you for providing me with every opportunity in life, for your emotional and financial support, and for your faith in my ability to achieve.

Most importantly, I thank my Heavenly Father for all the blessings I have received in my life.

## TABLE OF CONTENTS

<b>DECLARATION</b>	ii
<b>ABSTRACT</b>	iii
<b>OPSOMMING</b>	v
<b>ACKNOWLEDGEMENTS</b>	vii
<b>TABLE OF CONTENTS</b>	viii
<b>LIST OF TABLES</b>	xiv
<b>LIST OF FIGURES</b>	xv
<b>ABBREVIATIONS</b>	xxiv

## CHAPTER 1

### INTRODUCTION

<b>1.1 Chemical Communication in Mammals</b>	1
1.2.1 Semiochemical compounds	2
1.2.2 Production of semiochemicals	4
1.2.3 Detection of semiochemicals	5
1.2.3.1 The main olfactory system	6
1.2.3.2 The accessory olfactory system	6
<b>1.2 Kin Recognition</b>	7
1.2.1 General	7
1.2.2 Mammalian semiochemicals involved in kin recognition	8
1.2.3 Proteins involved in kin recognition	11
<b>1.3 The Domestic Sheep, <i>Ovis aries</i></b>	12
1.3.1 Background	12
1.3.2 Maternal behaviour and lamb survival	12
1.3.3 The olfactory system and offspring recognition in sheep	13
1.3.4 Amniotic fluid and ewe–lamb bond formation	14
1.3.5 Lamb fostering using odour manipulation	15

1.3.6	The use of semiochemicals in sheep husbandry	16
<b>1.4</b>	<b>Motivation and Aims of the Study</b>	17
<b>References</b>		19

## **CHAPTER 2**

### **EXPERIMENTAL**

<b>2.1</b>	<b>General</b>	28
<b>2.2</b>	<b>Sample Collection and Preparation</b>	28
<b>2.3</b>	<b>Analytical Methods</b>	30
2.3.1	Gas chromatographic analysis	30
2.3.2	Gas chromatographic-mass spectrometric analysis	30
2.3.3	Nuclear magnetic resonance spectrometry	31
2.3.4	Sampling methods	32
2.3.4.1	Conventional sample extraction	32
2.3.4.2	Solid phase micro-extraction headspace sampling	32
2.3.4.3	Sample enrichment probe headspace sampling	33
2.3.4.4	Analyses of synthesised standards and retention time comparison	34
<b>2.4</b>	<b>Dimethyl Disulphide Derivatisation</b>	35
<b>2.5</b>	<b>Determination of Absolute Configuration of Chiral Compounds</b>	35
<b>2.6</b>	<b>Protein Identification</b>	35
2.6.1	One-dimensional gel electrophoresis	36
2.6.2	In-gel tryptic digestion	37
2.6.3	Electrospray ionisation time-of-flight mass spectrometry	38
2.6.4	Anion-exchange chromatography	39
2.6.5	Ligand identification	39
2.6.6	Proteolysis	40
<b>2.7</b>	<b>Statistical Analysis</b>	40
<b>2.8</b>	<b>Bioassays</b>	40

<b>2.9 Synthesis of Reference Compounds</b>	42
2.9.1 Preparation of 2,6,10,14-tetramethylhexadecane	43
2.9.2 Preparation of branched primary alcohols	43
2.9.3 Preparation of 2-pentadecanol	45
2.9.4 Preparation of 6,10,14-trimethyl-2-pentadecanol	45
2.9.5 Preparation of aldehydes	46
2.9.6 Preparation of methyl ketones	48
2.9.7 Preparation of 6,10-dimethyl-2-undecanone	49
<b>Plate 2.1</b>	50
<b>References</b>	51

## CHAPTER 3

### CHEMICAL CHARACTERISATION OF THE COMPOUNDS PRESENT IN THE WOOL AND THE AMNIOTIC FLUID OF DÖHNE MERINO SHEEP, *OVIS ARIES*

<b>3.1 Chemical Characterisation of the Volatile Organic Fractions of the Wool and the Amniotic Fluid</b>	54
3.1.1 Introduction	54
3.1.2 Analytical methods	54
3.1.2.1 Gas chromatography-mass spectrometry	54
3.1.2.2 High resolution gas chromatography-mass spectrometry	55
3.1.2.3 Enantioselective gas chromatography-mass spectrometry	56
3.1.2.4 Determination of double bond positions	57
3.1.3 Chemical characterisation of the volatile organic fraction of the wool samples	58
3.1.3.1 Introduction	58
3.1.3.2 Sample collection and preparation	60
3.1.3.3 Sampling techniques	61
a) Solid-phase microextraction	61
b) Headspace enrichment with the sample enrichment probe technique	62



c)	Conventional sample extraction	66
3.1.4	Chemical characterisation of the volatile organic fraction of the amniotic fluid	67
3.1.4.1	Introduction	67
3.1.4.2	Sample collection and preparation	67
3.1.4.3	Sampling techniques	68
3.1.5	Structural elucidation of the constituents of cranial wool and amniotic fluid of the experimental animals	69
3.1.5.1	Saturated hydrocarbons	71
3.1.5.2	Unsaturated hydrocarbons	72
3.1.5.3	Unbranched primary alcohols	73
3.1.5.4	Branched primary alcohols	74
3.1.5.5	Unbranched aldehydes	75
3.1.5.6	Branched aldehydes	79
3.1.5.7	Unsaturated aldehydes	81
3.1.5.8	Aromatic aldehydes	81
3.1.5.9	Methyl ketones	82
3.1.5.10	Branched methyl ketones	82
3.1.5.11	Ethyl ketones	83
3.1.5.12	Unbranched carboxylic acids	84
3.1.5.13	Branched carboxylic acids	86
3.1.5.14	Unsaturated carboxylic acids	87
3.1.5.15	Esters	88
3.1.5.16	$\gamma$ -Lactones	89
3.1.5.17	Constituents with terpenoid character	90
3.1.5.18	Nitrogen containing constituents	92
3.1.5.19	Miscellaneous	94
3.1.5.20	Tentatively identified constituents and unidentified constituents	95
3.1.6	Conclusions regarding the qualitative composition of the wool and the amniotic fluid samples	98

<b>3.2 Characterisation of the Wool-Associated and the Amniotic Fluid Proteins</b>	109
3.2.1 Introduction	109
3.2.2 Sample collection and preparation	110
3.2.3 Determination of the composition of the protein fraction of the amniotic fluid	111
3.2.3.1 Strong anion exchange chromatography and ligand identification	113
3.2.4 Determination of the composition of the wool-associated proteins	115
3.2.5 Conclusions	116
<b>Plate 3.1</b>	119
<b>Plate 3.2</b>	120
<b>References</b>	121
<b>Total Ion Chromatograms and Mass Spectra</b>	130

## CHAPTER 4

### QUANTITATIVE ANALYSES OF THE COMPOUNDS PRESENT IN THE AMNIOTIC FLUID OF THE DÖHNE MERINO EWES AND THE WOOL OF THEIR LAMBS

<b>4.1 Quantitative Analysis of the Volatile Organic Fraction of the Wool Samples</b>	156
4.1.1 Introduction	156
4.1.2 Quantitative composition	159
4.1.2.1 Wool from day-old lambs	160
4.1.2.2 Wool from week-old lambs	169
4.1.2.3 Wool from Döhne Merino ewes	179
4.1.3 Statistical analysis	183
4.1.3.1 Genetic influence on the odour profile of the twin lambs	183
4.1.3.2 Changes in the lamb odour profiles over a one-week period	192
4.1.4 Conclusions regarding the quantitative composition of the wool	195
<b>4.2 Quantitative Analysis of the Volatile Organic Fraction of the Amniotic Fluid</b>	197
4.2.1 Introduction	197

4.2.2	Quantitative composition	197
4.2.3	Statistical analysis	202
4.2.3.1	Comparison of the amniotic fluid composition collected from twin lambs	202
4.2.3.2	Difference between the amniotic fluid composition of the first- and second-born lambs	205
4.2.4	Conclusions regarding the quantitative composition of the amniotic fluid	208
<b>References</b>		212

## **CHAPTER 5**

### **BIOASSAY**

<b>5.1</b>	<b>Introduction</b>	214
<b>5.2</b>	<b>Bioassays using Synthetic Mixtures</b>	215
<b>5.3</b>	<b>Bioassays using Different Odour-Transfer Techniques</b>	217
5.3.1	Odour-impregnated jackets	217
5.3.2	Wool of a lamb	219
5.3.3	Extracts of the wool of lambs	219
<b>5.4</b>	<b>Conclusions</b>	222
<b>Plate 5.1</b>		225
<b>References</b>		226

## **CHAPTER 6**

<b>CONCLUDING REMARKS AND OUTLOOK</b>	227
---------------------------------------	-----

## LIST OF TABLES

Table 3.1:	VOCs of the amniotic fluid of Döhne Merino ewes and the cranial wool of their lambs collected during the lambing seasons of 2007 and 2009.	101
Table 3.2:	VOCs present in the amniotic fluid of Döhne Merino ewes and the cranial wool of their lambs collected during the lambing seasons of 2007 and 2009 identified with HRMS.	106
Table 3.3:	Enantiomeric composition of chiral constituents of the wool and the amniotic fluid determined by GC-MS using columns C and D.	109
Table 3.4:	Proteins in the amniotic fluid of Döhne Merino ewes and in the wool of their lambs.	118
Table 4.1:	Ewe and lamb sample groups of 2007 and 2009.	158
Table 4.2:	Temporal changes in the percentage peak area of wool volatiles collected on days 1 and 7 after birth from lambs born during the lambing seasons of 2007 and 2009.	178
Table 4.3:	Average masses (ng) in which the VOCs are present in the amniotic fluid and the wool collected from Döhne Merino lambs during the lambing seasons of 2007 and 2009.	209

## LIST OF FIGURES

Fig. 1.1.	Classification of chemical signals employed in animal chemical communication.	3
Fig. 1.2.	Examples of mammalian pheromones (Albone, 1984: 77; Burger, 2005b; Burger <i>et al.</i> , 2008; Brennan and Keverne, 2004; Wyatt, 2003: 5)	3
Fig. 1.3.	Exocrine glands in mammals: (A) Mongolian gerbil ( <i>Meriones unguiculatus</i> ) cheek gland, (B) human skin (Bradbury and Vehrencamp, 1998: 284).	4
Fig. 1.4.	Functional and anatomical segregation of the main olfactory system in the rat (Dulac and Torello, 2003).	6
Fig. 1.5.	Functional and anatomical segregation of the accessory olfactory system in the rat (Dulac and Torello, 2003).	7
Fig. 1.6.	Comparison of the mean molecular weight of sex, recognition and territorial marking pheromones in five orders of mammals (Alberts, 1992).	9
Fig. 2.1.	Sample allocation of the amniotic fluid for GC-MS analysis.	29
Fig. 2.2.	Sample allocation of wool for GC-MS analysis.	29
Plate 2.1a.	Collection of amniotic fluid from a newborn lamb using a stainless steel spoon.	50
Plate 2.1b.	Collection of amniotic fluid in an Eppendorf tube for protein analyses.	50
Plate 2.1c.	Collection of wool samples.	50
Plate 2.1d.	SEP50 installed in a 50-ml glass bottle containing amniotic fluid.	50
Plate 2.1e.	SEP50 installed in a 50-ml glass bottle containing lamb's wool.	50
Plate 2.1f.	SEP50 installed in a 8-ml glass bottle containing lamb's wool.	50
Fig. 3.1.	TIC obtained by GC-EIMS analysis of the VOCs extracted with SEP50 from the wool collected from lamb US-2007-0638.	55
Fig. 3.2.	TIC obtained by GC-MS analysis of the VOCs extracted with TBME of the amniotic fluid collected from the body of lamb US-2007-0638.	55
Fig. 3.3.	TIC obtained by GC-MS analysis of the VOCs collected by SPME from the wool of lamb US-2007-0133.	61
Fig. 3.4.	TIC obtained by GC-MS analysis of the VOCs collected by SEP50 from the wool of lamb US-2007-0133.	62

Fig. 3.5.	TIC obtained by GC-MS analysis of the VOCs collected with a SEP50 over 2.75 h at 40 °C from <i>ca.</i> 200 mg of the wool of lamb US-2007-0046.	63
Fig. 3.6.	TIC obtained by GC-MS analysis of the VOCs collected with a SEP50 for 5 min at 145 °C from 70 mg of the wool of lamb US-2007-0126.	64
Fig. 3.7.	TIC obtained by GC-MS analysis of the VOCs collected with a SEP50 for 5 min at 100 °C from 70 mg of the wool of lamb US-2007-0416.	65
Fig. 3.8.	TIC obtained by GC-MS analysis of the VOCs collected with a SEP50 for 5 min at 70 °C from 70 mg of the wool of lamb US-2007-0416.	65
Fig. 3.9.	TIC obtained by GC-MS analysis of the VOCs collected with a SEP50 for 60 min at 70 °C from 70 mg of the wool of a lamb US-2007-0050.	65
Fig. 3.10.	TIC obtained by GC-MS analysis of the TBME extract of the wool of lamb US-2007-0046.	66
Fig. 3.11.	TIC obtained by GC-MS analysis of the VOCs collected with a SEP50 at 40 °C of the amniotic fluid collected from the lamb US-2007-526.	68
Fig. 3.12.	TIC of the headspace of the wool of lamb US-2007-0050.	130
Fig. 3.13.	TIC of the amniotic fluid extract collected off the body of lamb US-2007-0050.	131
Fig. 3.14.	The EI mass spectrum of nonane ( <b>C10</b> ).	132
Fig. 3.15.	The EI mass spectrum of dodecane ( <b>C36</b> ).	132
Fig. 3.16.	The EI mass spectrum of 1-tridecene ( <b>C44</b> ).	132
Fig. 3.17.	The EI mass spectrum of 1-hexacosene ( <b>C120</b> ).	133
Fig. 3.18.	The EI mass spectrum of DMDS adduct of 1-tridecene ( <b>C44</b> ).	133
Fig. 3.19.	The EI mass spectrum of 1-octanol ( <b>C25</b> ).	133
Fig. 3.20.	The EI mass spectrum of 1-eicosanol ( <b>C114</b> ).	134
Fig. 3.21.	The EI mass spectrum of 4-methyl-1-pentanol ( <b>C6</b> ).	134
Fig. 3.22.	The EI mass spectrum of heptanal ( <b>C8</b> ).	134
Fig. 3.23.	The EI mass spectrum of heptadecanal ( <b>C101</b> ).	135
Fig. 3.24.	The EI mass spectrum of 3-methylpentanal ( <b>C1</b> ).	135
Fig. 3.25.	The EI mass spectrum of 15-methylhexadecanal ( <b>C97</b> ).	135
Fig. 3.26.	The EI mass spectrum of ( <i>E</i> )-2-undecenal ( <b>C50</b> ).	136
Fig. 3.27.	The EI mass spectrum of DMDS adduct of ( <i>E</i> )-2-undecenal ( <b>C50</b> ).	136
Fig. 3.28.	The EI mass spectrum of benzaldehyde ( <b>C13</b> ).	136

Fig. 3.29.	The EI mass spectrum of phenylacetaldehyde ( <b>C22</b> ).	137
Fig. 3.30.	The EI mass spectrum of 2-decanone ( <b>C34</b> ).	137
Fig. 3.31.	The EI mass spectrum of 2-pentadecanone ( <b>C79</b> ).	137
Fig. 3.32.	The EI mass spectrum of 6-methyl-2-heptanone ( <b>C14</b> ).	138
Fig. 3.33.	The EI mass spectrum of 3-methyl-2-undecanone ( <b>C47</b> ).	138
Fig. 3.34.	The EI mass spectrum of 3-octanone ( <b>C16</b> ).	138
Fig. 3.35.	The EI mass spectrum of 3-decanone ( <b>CP4</b> ).	139
Fig. 3.36.	The EI mass spectrum of acetic acid ( <b>CP3</b> ).	139
Fig. 3.37.	The EI mass spectrum of propanoic acid ( <b>CP5</b> ).	139
Fig. 3.38.	The EI mass spectrum of nonanoic acid ( <b>C42</b> ).	140
Fig. 3.39.	The EI mass spectrum of tetradecanoic acid ( <b>C85</b> ).	140
Fig. 3.40.	The EI mass spectrum of hexadecanoic acid ( <b>C104</b> ).	140
Fig. 3.41.	The EI mass spectrum of heptadecanoic acid ( <b>C107</b> ).	141
Fig. 3.42.	The EI mass spectrum of 12-methyltridecanoic acid ( <b>C82</b> ).	141
Fig. 3.43.	The EI mass spectrum of 14-methylpentadecanoic acid ( <b>C102</b> ).	141
Fig. 3.44.	The EI mass spectrum of 15-methylhexadecanoic acid ( <b>C106</b> ).	142
Fig. 3.45.	The EI mass spectrum of ( <i>Z</i> )-9-hexadecenoic acid ( <b>C103</b> ).	142
Fig. 3.46.	The EI mass spectrum of ( <i>Z</i> )-9-octadecenoic acid ( <b>C111</b> ).	142
Fig. 3.47.	The EI mass spectrum of DMDS adduct of ( <i>Z</i> )-9-hexadecenoic acid ( <b>C103</b> ).	143
Fig. 3.48.	The EI mass spectrum of DMDS adduct of ( <i>Z</i> )-9-octadecenoic acid ( <b>C111</b> ).	143
Fig. 3.49.	The EI mass spectrum of ( <i>Z, Z</i> )-9,12-octadecadienoic acid ( <b>C110</b> ).	143
Fig. 3.50.	The EI mass spectrum of ethyl tetradecanoate ( <b>C88</b> ).	144
Fig. 3.51.	The EI mass spectrum of isopropyl tetradecanoate ( <b>C92</b> ).	144
Fig. 3.52.	The EI mass spectrum of dodecan-4-olide ( <b>C76</b> ).	144
Fig. 3.53.	The EI mass spectrum of 6,10-dimethyl-2-undecanone ( <b>C56</b> ).	145
Fig. 3.54.	The EI mass spectrum of ( <i>5E</i> )-6,10-dimethyl-5,9-undecadien-2-one ( <b>C60</b> ).	145
Fig. 3.55.	The EI mass spectrum of 2,6,10,14-tetramethylhexadecane ( <b>C91</b> ).	145
Fig. 3.56.	The EI mass spectrum of 6,10,14-trimethyl-2-pentadecanone ( <b>C93</b> ).	146

Fig. 3.57.	The EI mass spectrum of 6,10,14-trimethyl-2-pentadecanol ( <b>C95</b> ).	146
Fig. 3.58.	The EI mass spectrum of squalene ( <b>C122</b> ).	146
Fig. 3.59.	The EI mass spectrum of 2-methylpyrimidine ( <b>C5</b> ).	92 & 147
Fig. 3.60.	The EI mass spectrum of 2,5-dimethylpyrimidine ( <b>C11</b> ).	92 & 147
Fig. 3.61.	Methylpyrazine (NIST).	93
Fig. 3.62.	2-Methylpyrimidine (NIST).	93
Fig. 3.63.	2,5-Dimethylpyrazine (NIST).	93
Fig. 3.64.	2,6-Dimethylpyrazine (NIST).	93
Fig. 3.65.	2,5-Dimethylpyrimidine (NIST).	93
Fig. 3.66.	4,6-Dimethylpyrimidine (NIST).	93
Fig. 3.67.	The EI mass spectrum of N-methyl-2-piperidinone ( <b>C29</b> ).	147
Fig. 3.68.	The EI mass spectrum of 3-octanol ( <b>CP2</b> ).	148
Fig. 3.69.	The EI mass spectrum of dimethyl sulfone ( <b>C9</b> ).	148
Fig. 3.70.	The EI mass spectrum of 2-pentylfuran ( <b>C18</b> ).	148
Fig. 3.71.	The EI mass spectrum of benzoic acid ( <b>C32</b> ).	149
Fig. 3.72.	The EI mass spectrum of cholest-5-en-3 $\beta$ -ol ( <b>C124</b> ).	149
Fig. 3.73.	The EI mass spectrum of 3-ethyl-4-methyl-1H-pyrrole-2,5-dione ( <b>C37</b> ).	149
Fig. 3.74.	The EI mass spectrum of 3-ethyl-4-methyl-pyrrolidine-2,5-dione ( <b>C38</b> ).	150
Fig. 3.75.	The EI mass spectrum of 3-methyl-4-vinyl-1H-pyrrole-2,5-dione ( <b>C39</b> ).	150
Fig. 3.76.	The EI mass spectrum of unidentified constituent <b>C96</b> .	150
Fig. 3.77.	The EI mass spectrum of unidentified constituent <b>C84</b> .	151
Fig. 3.78.	The EI mass spectrum of unidentified constituent <b>C3</b> .	151
Fig. 3.79.	The EI mass spectrum of unidentified constituent <b>C7</b> .	151
Fig. 3.80.	The EI mass spectrum of unidentified constituent <b>C12</b> .	152
Fig. 3.81.	The EI mass spectrum of unidentified constituent <b>C21</b> .	152
Fig. 3.82.	The EI mass spectrum of unidentified constituent <b>C49</b> .	152
Fig. 3.83.	The EI mass spectrum of unidentified constituent <b>C52</b> .	153
Fig. 3.84.	The EI mass spectrum of unidentified constituent <b>C59</b> .	153



Fig. 3.85.	The EI mass spectrum of unidentified constituent <b>C63</b> .	153
Fig. 3.86.	The EI mass spectrum of unidentified constituent <b>C64</b> .	154
Fig. 3.87.	The EI mass spectrum of unidentified constituent <b>C70</b> .	154
Fig. 3.88.	The EI mass spectrum of unidentified constituent <b>C74</b> .	154
Fig. 3.89.	The EI mass spectrum of unidentified constituent <b>C83</b> .	155
Fig. 3.90.	The EI mass spectrum of unidentified constituent <b>C121</b> .	155
Fig. 3.91.	The EI mass spectrum of unidentified constituent <b>C123</b> .	155
Fig. 3.92.	Amniotic fluid proteins separated on 8% SDS-PAGE gel (200 µg).	110
Fig. 3.93.	Wool-associated proteins separated on 8% SDS-PAGE gel (200 µg).	110
Fig. 3.94.	Amino acid sequence of serum albumin ( <i>Ovis aries</i> ). Peptides identified in the amniotic fluid are indicated in bold letters and the areas of high conservancy are <u>underlined</u> .	112
Fig. 3.95.	Amino acid sequence of immunoglobulin lambda light-chain (constant region) ( <i>Ovis aries</i> ). Peptides identified in the amniotic fluid are indicated in bold letters.	113
Fig. 3.96.	Separation of amniotic fluid proteins by anion-exchange chromatography using a HiTrap Q column and a NaCl gradient.	114
Fig. 3.97.	Amniotic fluid proteins separated on 10% SDS-PAGE gel (200 µg).	114
Fig. 3.98.	Amino acid sequence of serum albumin ( <i>Ovis aries</i> ). Peptides identified from the wool samples are indicated in bold letters and the areas of high conservancy are <u>underlined</u> .	115
Fig. 3.99.	Amino acid sequence of a foetal haemoglobin beta chain ( <i>Ovis aries</i> ). Peptides identified from the wool samples are indicated in bold letters.	115
Fig. 3.100.	Amino acid sequence of the immunoglobulin lambda chain (C region) ( <i>Ovis aries</i> ). Peptides identified from the wool samples are indicated in bold letters.	116
Plate 3.1a.	Ewe licking the amniotic fluid off her newborn lamb.	119
Plate 3.1b.	Ewe eating the foetal membrane and amniotic fluid off her newborn lamb.	119
Plate 3.1c.	Ewe sniffing at the nose and mouth of a lamb.	119
Plate 3.1d.	Ewe sniffing at the neck of an alien lamb.	119
Plate 3.1e.	Ewe sniffing at the nose of a alien lamb.	119

Plate 3.1f.	Ewe sniffing at the body of a lamb.	119
Plate 3.2a.	Ewe sniffing at a lamb's hind quarters.	120
Plate 3.2b.	Ewe sniffing at an alien lamb's neck.	120
Plate 3.2c.	Ewe sniffing at an alien lamb's hind quarters.	120
Plate 3.2d.	Ewe sniffing at the neck of an alien lamb.	120
Plate 3.2e.	Ewe sniffing at an alien lamb's head.	120
Fig. 4.1.	Quantitative composition of the wool volatiles collected from day-old Döhne Merino lambs during the lambing season of 2007.	162
Fig. 4.2.	Quantitative composition of the wool volatiles collected from day-old Döhne Merino lambs during the lambing season of 2009.	162
Fig. 4.3.	Volatile constituents present in percentages larger than 1% in the wool of day-old lambs born during the lambing seasons of 2007 (dark grey) and 2009 (light grey). The constituents are numbered on the x-axis.	164
Fig. 4.4.	Volatile constituents present in percentages smaller than 1% in the wool of day-old lambs born during the lambing seasons of 2007 (dark grey) and 2009 (light grey). The constituents are numbered on the x-axis. Part 1 and 2.	165 & 166
Fig. 4.5.	TIC obtained by GC-MS analysis of the headspace of the wool of Döhne Merino lamb US-2007-0105.	167
Fig. 4.6.	TIC obtained by GC-MS analysis of the headspace of the wool of Döhne Merino lamb US-2007-0106.	167
Fig. 4.7.	TIC obtained by GC-MS analysis of the headspace of the wool of Döhne Merino lamb US-2007-0114.	167
Fig. 4.8.	TIC obtained by GC-MS analysis of the headspace of the wool of Döhne Merino lamb US-2007-0115.	167
Fig. 4.9.	Quantitative comparison (percentage peak area) of the wool volatiles of day-old twin lambs US-2007-0224 (black) and US-2007-0225 (grey).	168
Fig. 4.10.	Quantitative comparison (percentage peak area) of the wool volatiles of the unrelated lambs US-2007-0224 (black) and US-2007-0050 (grey).	168
Fig. 4.11.	Quantitative composition (percentage peak areas) of the wool volatiles collected from Döhne Merino lambs during the lambing season of 2009.	169
Fig. 4.12.	TIC obtained by GC-MS analysis of the headspace of the wool of the day-old Döhne merino lamb US-2007-0516.	170
Fig. 4.13.	TIC obtained by GC-MS analysis of the headspace of the wool of the week-old Döhne merino lamb US-2007-0516.	170

- Fig. 4.14. Quantitative comparison (percentage peak area) of the wool volatiles of the lamb US-2007-0224 collected on day 1 (black) and day 7 (grey) after birth during the lambing season of 2007. 170
- Fig. 4.15. Comparison of the percentage peak areas of the wool volatile present in percentages larger than 1% in the wool of day-old (black) and week-old lambs (grey) born during the lambing season of 2007. The constituents are numbered on the x-axis. 172
- Fig. 4.16. Comparison of the percentage peak areas of the wool volatile present in percentages smaller than 1% in the wool of day-old (black) and week-old lambs (grey) born during the lambing season of 2007. The constituents are numbered on the x-axis. Part 1 and 2. 173 & 174
- Fig. 4.17. Changes in percentage peak areas of the wool volatiles collected from lamb US-2009-0736 on day 1 (blue), day 5 (yellow) and day 7 (green) after birth during the lambing season of 2009. The constituents are numbered on the x-axis. Part 1 and 2. 176 & 177
- Fig. 4.18. Comparison of the percentage peak areas of the wool volatiles collected from the head of lamb US-2009-0736 (blue), and the wool collected from the head (yellow) and the abdomen (green) of this lamb's mother during the lambing season of 2009. Compound numbers are indicated on the x-axis of the graph. 181
- Fig. 4.19. Comparison of the percentage peak areas of the wool volatiles collected from the heads of the unrelated lambs US-2009-0736 (blue), US-2009-0520 (yellow) and US-2009-0548 (green) born during the lambing season of 2009. Compound numbers are indicated on the x-axis of the graph. 182
- Fig 4.20. Schematic representation of how the *P*-value was obtained (Le Roux and Lubbe, 2009). 185
- Fig 4.21. Permutation distribution showing the differences in the composition of the wool volatiles collected from day-old twin lambs compared to the rest of the group of lambs. 186
- Fig. 4.22. PCA biplot for the wool volatiles collected from day-old lambs during the lambing season of 2007. 188
- Fig. 4.23. PCA biplot displaying the crowded area in Fig. 4.22. Lambs possessing similar odour profiles are encircled. 188
- Fig. 4.24. PCA biplot for the wool volatiles collected from day-old lambs during the lambing season of 2009. 190
- Fig. 4.25. TIC of the SEP enriched wool volatiles of the day-old lamb US-2007-0103 of ewe US-2002-0424. 191
- Fig. 4.26. TIC of the SEP enriched wool volatiles of the day-old lamb US-2009-0330 of ewe US-2002-0424. 191
- Fig. 4.27. PCA biplot for the wool volatiles collected from day-old lambs during the lambing seasons of 2007 and 2009. 192

Fig. 4.28.	PCA biplot for the wool volatiles collected from week-old lambs during the lambing season of 2007.	193
Fig. 4.29.	PCA biplot for the wool volatiles collected from day-old lambs (blue) and from the same lambs a week after birth (green).	195
Fig. 4.30.	Quantitative composition (average percentage peak areas) of the volatile organic fraction of the amniotic fluid collected from the bodies of Döhne Merino lambs during the lambing season of 2007.	198
Fig. 4.31.	Volatile constituents present in percentages larger than 1% of the volatile organic material extracted from the amniotic fluid collected from the bodies of newborn lambs during the lambing season of 2007. Constituents are shown in order of elution from Column A and constituent numbers are indicated on the x-axis.	200
Fig. 4.32.	Volatile constituents present in percentages smaller than 1% of the volatile organic material extracted from the amniotic fluid collected from the bodies of newborn lambs during the lambing season of 2007. Constituents are shown in order of elution from Column A and constituent numbers are indicated on the x-axis.	201
Fig. 4.33.	TIC of the TBME extract of the amniotic fluid collected from the body of lamb US-2007-0049.	203
Fig. 4.34.	TIC of the TBME extract of the amniotic fluid collected from the body of lamb US-2007-0050.	203
Fig. 4.35.	TIC of the TBME extract of the amniotic fluid collected from the body of lamb US-2007-0431.	203
Fig. 4.36.	TIC of the TBME extract of the amniotic fluid collected from the body of lamb US-2007-0432.	203
Fig. 4.37.	Permutation distribution showing the differences in the composition of the amniotic fluid of twin lambs compared to the rest of the group.	204
Fig. 4.38.	PCA biplot of the amniotic fluid dataset displaying only the axes that best represent the variation in the amniotic fluid composition.	205
Fig. 4.39.	Section of the PCA biplot depicted in Fig. 4.38 displaying the lambs in the concentrated area.	205
Fig. 4.40.	Newborn lamb covered with mostly colourless amniotic fluid, with a slight discolouration at the lamb's hind quarters.	206
Fig. 4.41.	Newborn lamb covered with yellow amniotic fluid.	206
Fig. 4.42.	95%-Bagplot comparing the amniotic fluid of the first-born lambs (red) and second-born lambs (green). Squares and dots represent lambs and are indicated by the last three digits of the lambs' US numbers.	207

Fig. 4.43.	95%-Bagplot comparing the amniotic fluid of the first-born (red) and second-born (green). Five aldehydes have been added to the dataset depicted in Fig. 4.42. The positions of three twins are shown with black intra-twin connecting lines.	207
Fig. 5.1.	TIC obtained by GC-MS analysis of the headspace volatiles SEP-enriched at 40 °C from a jacket worn by a Döhne Merino lamb and used in a successful fostering experiment.	218
Fig. 5.2.	TIC of a typical pentane extract of the wool of lamb US-2009-0654.	221
Fig. 5.3.	TIC of a typical TBME extract of the wool of lamb US-2007-0046.	222
Plate 5.1a.	Fitting a lamb with a diaper and with thin, stretchable plastic film.	225
Plate 5.1b.	Fitting a lamb with a hoodless fleece jacket.	225
Plate 5.1c.	Ewe investigating an alien lamb fitted with a hooded fleece jacket.	225
Plate 5.1d.	Spraying the fleece jacket of an alien lamb with a mixture of synthetic compounds.	225
Plate 5.1e.	Ewe investigating alien lamb fitted with a jacket made from brown paper.	225
Plate 5.1f.	Alien lamb accepted at the udder.	225

## ABBREVIATIONS

AOB	: Accessory olfactory bulb
AOS	: Accessory olfactory system
BLAST	: Basic local alignment search tool
°C	: Degrees Celsius
CDCl <sub>3</sub>	: Deuteriochloroform
DCM	: Dichloromethane
DMDS	: Dimethyl disulphide
DTT	: Dithiothreitol
EDTA	: Ethylenediaminetetra-acetic acid
EI	: Electron impact
EIMS	: Electron impact mass spectrometer Electron impact mass spectrometric
ESI-Q-TOF-MS	: Electrospray ionization quadrupole time-of-flight mass spectrometer
FID	: Flame ionisation detector
GC	: Gas chromatograph Gas chromatographic
GC-CIMS	: Gas chromatograph-chemical ionisation mass spectrometer Gas chromatographic-chemical ionisation mass spectrometric
GC-HRMS	: Gas chromatography-high resolution mass spectrometry Gas chromatographic-high resolution mass spectrometric
GC-LRMS	: Gas chromatography-low resolution mass spectrometry Gas chromatographic-low resolution mass spectrometric
GC-MS	: Gas chromatography-mass spectrometry Gas chromatographic-mass spectrometric
GC-TOF-HRMS	: Gas chromatography-time-of-flight high resolution mass spectrometry Gas chromatographic-time-of-flight high resolution mass spectrometric
HR	: High resolution
i.d.	: Inner diameter
IS	: Internal standard
LC-MS-MS	: Liquid chromatography-mass spectrometry-mass spectrometry
LECUS	: Laboratory for Ecological Chemistry, Stellenbosch University
LPLC	: Low pressure liquid chromatography
MANOVA	: Multivariate analysis of variance
MHC	: Major histocompatibility complex
MOB	: Main olfactory bulb
MOE	: Main olfactory epithelium

MOS	: Main olfactory system
MOWSE	: Molecular weight search
MS/MS	: Tandem mass spectrometric
MUPs	: Major urinary proteins
NMR	: Nuclear magnetic resonance
OSNs	: Olfactory sensory neurons
PAEs	: Phthalic acid esters
PBPs	: Pheromone-binding proteins
PDMS	: Polydimethyl siloxane
PCA	: Principal component analysis
PCC	: Pyridium chlorochromate
PDMS	: Polydimethylsiloxane
Pt/C	: Platinum (10%) on activated charcoal
RI	: Retention index
rpm	: Revolutions per minute
RSD	: Relative standard deviation
SDS	: Sodium dodecyl sulphate
SDS-PAGE	: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	: Standard error of the mean
SEP	: Sample enrichment probe
SPME	: Solid phase micro-extraction
TBME	: <i>tert</i> -butyl methyl ether
TCA	: Trichloroacetic acid
TEMED	: N,N,N',N'-tetramethylethylenediamine
TIC	: Total ion chromatogram
Tris-HCl	: <i>tris</i> (hydroxymethyl)aminomethane
VA	: Vomeronasal amygdala
VNO	: Vomeronasal organ
VOCs	: Volatile organic constituents
VSNs	: Vomeronasal sensory neurons

# CHAPTER 1

## INTRODUCTION

### 1.1 Chemical Communication in Mammals

Animals' perception of the world around them is formed by gathering information using their senses, and this information is used for orientation, nourishment and reproduction. Highly social animals use complex interactions and their communication goes further than the necessary communication for reproduction. Using efficient communication, animals arrange themselves according to their relative status in their communities. The signals used by animals for communication have developed into diverse forms that are dependent on the environment in which they are used, as well as on the physiological equipment of the receiver and sender of the message. This led to the development of different forms of communication: chemical, auditory, visual and sensory communication. Of these forms of communication, chemical communication is the oldest and most important form of conveying information between living organisms. This type of communication is made possible by the release and perception of chemical compounds. Since the start of life on earth, single-cell organisms have possessed the ability to selectively discriminate between different chemical compounds required for their cellular metabolism (Bradbury and Vehrencamp, 1998: 279; Wyatt, 2005: 7–8). The importance of chemical communication has long been recognised, even before humanity realised that pheromones, or even molecules, exist.

Unravelling the chemical communication in higher vertebrates presents greater challenges than studying the chemical communication of insects. Unlike the complex molecules used by insects for their communication, mammals often make use of small, simple molecules, and they achieve complexity and selectivity by using a large number of constituents of different compound types that together act as a single pheromone. In some instances, selectivity is achieved by the interaction of small molecules with carrier proteins (Wyatt, 2005: 7–8).



---

---

Numerous practical applications have emerged resulted from the findings of animal behaviour and communication studies. Behavioural studies on mice have shown that chemical compounds are used to stimulate or inhibit ovulation, to synchronise menstrual cycles as well as induce miscarriages. Non-toxic pesticides have been identified and commercialised by the study of insect communication. Endangered species can be protected if the necessary information regarding their behaviour and social interactions, reproductive behaviour, communication and the animals' unique requirements for survival can be obtained through careful study (Bradbury and Vehrencamp, 1998: 6; Brennan and Keverne, 2004). Natural chemical compounds are increasingly used to influence the behaviour of domesticated animals, especially during reproductive processes, but they can only be utilised if they are isolated, identified and made commercially available (Wyatt, 2003: 251)

### 1.2.1 Semiochemical compounds

Pheromones were first defined by Karlson en Lüscher (1959) as substances, or mixtures of substances, secreted by an individual and received by another individual of the same species in which they elicit a specific reaction. The reactions produced by pheromones vary from long-term, irreversible physiological responses to specific instantaneous changes in behaviour. The term *pheromone* is derived from the Greek *pherein*, "to transfer" or "to carry", and *hormōn*, "to stimulate". Using a broader term, chemical constituents involved in animal communication are described as *semiochemical compounds*, from the Greek *semeion*, "to signal" (Law and Regnier, 1971). Pheromones are a subclass of semiochemicals, and are used for intraspecific communication. Wilson (1963) subdivided pheromones into "primer" and "releaser" pheromones according to the different behavioural patterns evoked by the respective pheromones. Releaser pheromones prompt an instantaneous behavioural response, whereas primer pheromones elicit a lasting physiological change in the endocrine and reproductive system of the receiver of the chemical message (Shorey, 1976: 2–4). Semiochemical compounds responsible for the interspecific transmission of information between different species are known as *allelochemical compounds*. Allelochemical compounds that are beneficial to the sender of the message are known as *allomonas*, whereas allelochemical compounds that are beneficial to the receiver of the message are known as

*kairomones* (Wyatt, 2003: 1–2). The semiochemical communication concept is summarised in Fig. 1.1 and some examples of mammalian semiochemicals and their physiological effects are shown in Fig. 1.2.

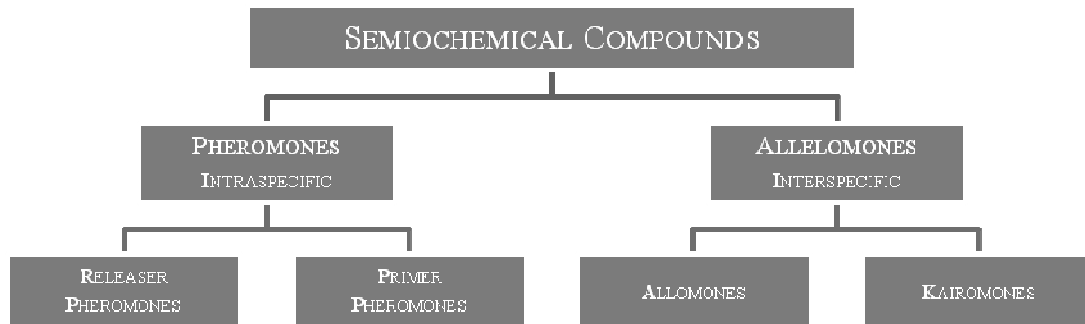


Fig. 1.1. Classification of chemical signals employed in animal chemical communication.

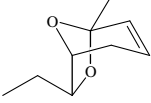
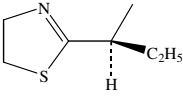
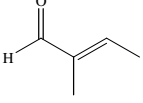
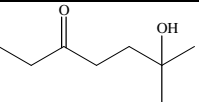
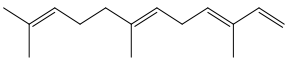
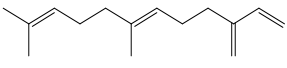
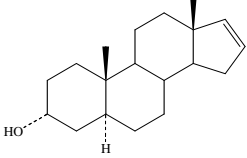
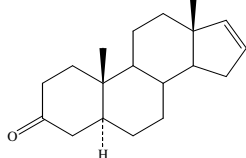
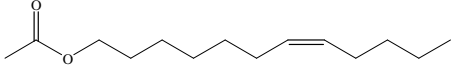
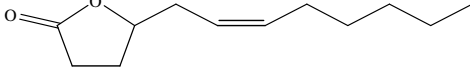
 3,4-Dehydro- <i>exo</i> -brevicommin Oestrus induction, inter-male aggression and synchronisation of menstrual cycles in female mice	 2- <i>sec</i> -Butyl-4,5-dihydrothiazole	 2-Methyl-2-butenal Nipple search response in rabbit pups
 6-Hydroxy-6-methyl-3-heptanone	 ( <i>E,E</i> )- $\alpha$ -Farnesene	 ( <i>E</i> )- $\beta$ -Farnesene
Puberty acceleration in female mice and inter-male aggression		
 5 $\alpha$ -Androst-16-en-3-ol	 5 $\alpha$ -Androst-16-en-3-one	
Sex pheromones present in the saliva of male boars		
 ( <i>Z</i> )-7-Dodecen-1-yl acetate Sex pheromone of female elephants inducing sexual behaviour in male elephants.	 ( <i>Z</i> )-6-Dodecen-4-olide Component of the tarsal hair scent of the male black-tailed deer, the interdigital secretion of the bontebok and the marking fluid of the male Bengal tiger.	

Fig. 1.2. Examples of mammalian pheromones (Albone, 1984: 77; Burger, 2005b; Burger *et al.*, 2008; Brennan and Keverne, 2004; Wyatt, 2003: 5)

## 1.2.2 Production of semiochemicals

Vertebrates and invertebrates have various glands that produce chemical compounds. Semiochemicals can either be produced by well-defined secretory glands that secrete their product outside of the animal's body, or the semiochemicals are excreted by body orifices and organs involved in the digestion and reproduction systems of the animal.

Endocrine glands release hormones into the bloodstream and control the body's internal metabolic processes, whereas exocrine glands are situated externally on the skin, or are situated internally with ducts leading to the exterior of the body. The function of exocrine glands can be the maintenance of the condition of the body's surface and/or the production of semiochemical constituents. Fat glands and sweat glands are the two types of exocrine glands found in mammals (Fig. 1.3). These glands possess well-defined structures that produce, store and secrete chemical compounds. The release of semiochemical products is usually accompanied by specific behavioural patterns, from which the function of the released chemical compound can be deduced (Bradbury and Vehrencamp, 1998: 283–284).

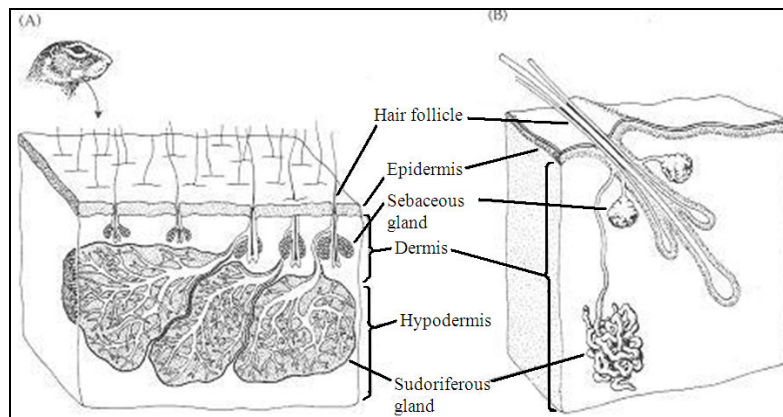


Fig. 1.3. Exocrine glands in mammals: (A) Mongolian gerbil (*Meriones unguiculatus*) cheek gland, (B) human skin (Bradbury and Vehrencamp, 1998: 284).

All bodily secretions could possibly act as semiochemical messengers. The production of chemical compounds that aid digestion is the primary function of glands associated with the digestive system. However, these compounds may also act as semiochemical messengers. For example, the saliva of male boars is the source of the

---

---

androgen derivatives, 5 $\alpha$ -androst-16-en-3-one and 5 $\alpha$ -androst-16-en-3-ol (Fig. 1.2). These pheromones are involved in the courtship of pigs (Bradbury and Vehrencamp, 1998: 284; Brennan and Keverne, 2004)

In some animals microorganisms are a source of semiochemicals. Many areas of the mammalian body surface are well suited for bacterial growth and bacteria produce a great number of different volatile metabolic waste products that could possibly act as semiochemicals (Albone, 1984: 135).

### 1.2.3 Detection of semiochemicals

The detection of semiochemicals is facilitated by either the main olfactory system (MOS) or the accessory olfactory system (AOS). The MOS detects volatile odours in air, whereas the AOS typically detects non-volatile cues sampled through direct physical contact (Dulac and Torello, 2003). The complex mixture of molecules constituting the airborne odour signature of an individual appears to contain few, if any, unique compounds (Brennan and Kendrick, 2006). It is not the complexity of a chemical message, but rather the relative ratio of volatile compounds in the odour profile that conveys the information about individual identity (Singer *et al.*, 1997; Schaefer *et al.*, 2002). The MOS is ideally suited for the recognition of profiles of airborne volatiles that characterise an individual's odour signature (Brennan and Kendrick, 2006). Lévy *et al.* (1995, 2004) credit the MOS for individual recognition. They found that lesioning of the olfactory bulbs or the olfactory epithelium causes loss of selectivity so that a ewe, for example, showed no preference in lamb selection. Maternal selectivity was, however, not affected when the vomeronasal nerve was cut. This implies that the semiochemicals involved in ewe–lamb recognition are mediated by the MOS. The perception of volatile compounds by the vomeronasal organ (VNO) located in the AOS, and the possible role that the VNO plays in individual recognition can, however, not be excluded since its role in pheromone detection in sheep has been shown (Booth, 2006; Johnston, 1998; Trinh and Storm, 2003). An alternative point of view is taken by Keller *et al.* (2009) and Restrepo *et al.* (2004), who are in agreement that both the MOS and the AOS are able to process partially overlapping sets of chemosignals and that these two systems complement each other during recognition. It is therefore clear

that the roles played by the MOS and the AOS in individual recognition are, at present, still controversial.

### 1.2.3.1 The main olfactory system

Olfactory sensory neurons (OSNs) form the main olfactory epithelium (MOE) that lines the upper part of the nasal cavity in mammals and this epithelium is specialised for the detection of volatile molecules (Dulac and Torello, 2003). Odour molecules are detected by the OSN receptor proteins and the olfactory signal is transmitted from the MOE to the main olfactory bulb (MOB). The signals are then relayed from the MOB to various parts of the brain, mainly the primary olfactory cortex (Fig. 1.4) (Dulac and Torello, 2003). Each OSN expresses only one receptor protein and the receptor protein detects only one particular class of odour molecules (Serizawa *et al.*, 2000).

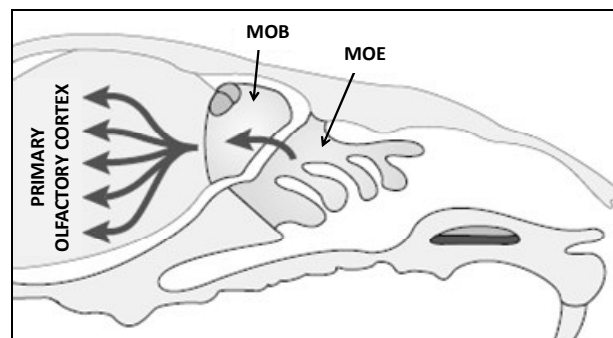


Fig. 1.4. Functional and anatomical segregation of the main olfactory system in the rat (Dulac and Torello, 2003).

### 1.2.3.2 The accessory olfactory system

In addition to the MOS, most mammals also possess a well-developed AOS, with the sensory receptors of the AOS located in the VNO. The AOS is considered to be specialised for detection of semiochemical compounds (Dulac and Torello, 2003). The vomeronasal sensory neurons (VSNs) differ from the OSNs since each VSN contains multiple receptor proteins and each odorant receptor recognises multiple odourants (Song *et al.*, 2008). Another difference between the MOS and the AOS is that the signals detected by the VNO are not relayed to the same areas in the brain as the signals detected by the MOS, but to the hypothalamus (Dulac and Torello, 2003). The VNO is a mucus-filled tubular organ situated

inside the nasal septum (Døving and Trotier, 1998). Detection of molecules by the VNO differs from that of the MOS; the vomeronasal sensory neurons are located away from the nasal airstream and a vascular pumping mechanism provides access of the stimulus to the VNO (Brennan and Keverne, 2004). This mechanism enables the VNO to detect relatively non-volatile molecules. These stimuli are detected by direct contact and specific behaviours, such as facial grooming. Flehmen may assist transport of substances into the VNO (Luo *et al.*, 2003). The vomeronasal system is, however, also capable of responding to volatile semiochemicals (Brennan and Keverne, 2004).

Fig. 1.5 illustrates the processing of semiochemical signals by the VNO in the rat. Odour molecules are detected by receptor proteins of the VSN situated in the VNO. The signal is relayed from the VNO to the accessory olfactory bulb (AOB), and from the AOB via the vomeronasal amygdale (VA) to the hypothalamus (Dulac and Torello, 2003).

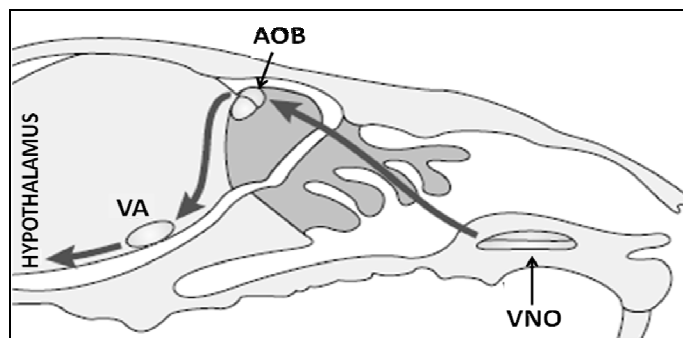


Fig. 1.5. Functional and anatomical segregation of the accessory olfactory system in the rat (Dulac and Torello, 2003).

## 1.2 Kin Recognition

### 1.2.1 General

The ability to recognise and respond to different individuals is important in all social structures, particularly among higher mammals that possess the ability to learn information about specific individuals and use this information to adjust their behaviour in subsequent interactions. Olfactory cues are used in the recognition of sex, individual identity and for species recognition. For mammals to have the ability to recognise kin, the individual must

---

---

possess a variety of abilities. First, an individual must be able to produce a cue by which it indicates its relatedness to the next individual and, second, the individual must be able to process this cue to determine relatedness and then act on this cue (Hepper and Cleland, 1999).

Odours used for kin recognition can be of environmental or genetic origin, as well as a combination of the two. Animals can recognise one another by three possible mechanisms. The first is learning by direct familiarisation, i.e. learning the characteristics of surrounding individuals; the second is by learning their own odour and comparing it with others for phenotype matching; and the third is by self-inspection, when they recognise an animal with the same characteristics. The odours implicated in parent–offspring recognition seem to be genetic and are produced by the individual itself. Recognition cues are usually learned through behavioural rules, and there is usually a sensitive period during which the chemical cues are learned (Wyatt, 2003: 103–105).

### 1.2.2 Mammalian semiochemicals involved in kin recognition

A wide variety of organic chemical compounds are used for chemical communication. The molecular composition of the signal is determined by the medium in which it is transmitted, namely water or air, and the manner in which the signal is transmitted, whether by diffusion, current or contact. Semiochemical compounds released into the air must be volatile (a compound's volatility is a function of its molecular size and composition). Volatile molecules display an upper size limit in terms of molecular weight, namely *ca.* 300, although most airborne odour molecules contain between five and twenty carbon atoms. Molecules containing more than twenty carbon atoms are energetically expensive to produce and have low diffusion rates, while molecules containing less than five carbon atoms are probably uncommon because they are too volatile and possess too few options for species-specific structural variants. Within this size range, compounds show great variation in shape and type of functional group. All the semiochemicals shown in Fig. 1.2 fit into this size range. Semiochemicals vary greatly in terms of functional group position, double bond position, and the occurrence of branches and rings in the carbon chain. Although these variations have minor effects on volatility, they greatly affect the shape of

the molecule and the molecular detection by receptor cells (Bradbury and Vehrencamp, 1998: 281–283).

Alberts (1992) has reported on the constraints on the design of chemical communication systems in terrestrial vertebrates. The constraints on semiochemicals used particularly for kin recognition will now be discussed briefly. Evolutionary selection is expected to favour the use of signalling strategies that convey messages efficiently and minimize energetic costs to the signalling animals. In order to be efficient transmitters of information about individuals in kin recognition, the semiochemicals involved should satisfy certain physical requirements. Semiochemicals used primarily for short-range kin recognition have to provide detailed information about individual identity, social status or reproductive condition, and should thus display sufficient molecular diversity to encode these complex messages. It is expected that semiochemicals used as recognition signals, as well as semiochemicals used for sex attraction, should have higher rates of diffusion that result in rapid rise times so that they can be detected easily and quickly. When comparing the molecular weights of semiochemicals used for sex attraction, recognition and territorial marking, it is clear that the molecular weights of those compounds used for sexual attraction and for individual recognition display lower molecular weights within mammalian orders as well as across mammalian orders, than semiochemicals used for territorial marking, as could be expected (Fig. 1.6).

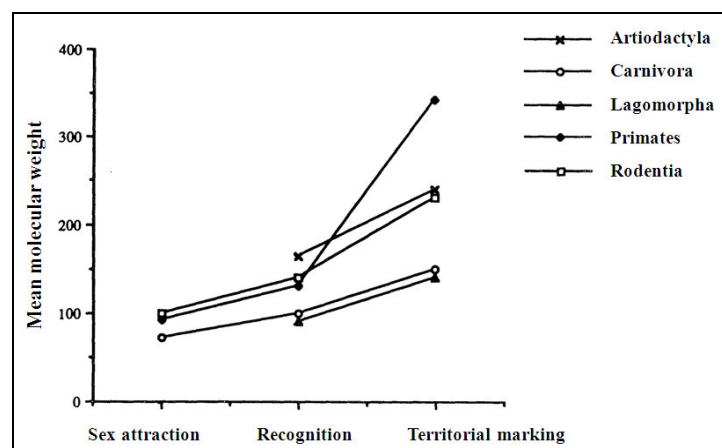


Fig. 1.6. Comparison of the mean molecular weight of sex, recognition and territorial marking pheromones in five orders of mammals (Alberts, 1992).



---

---

Mammals can prolong the persistence of signals by making use of carrier compounds that delay the diffusion of volatile constituents. Sebum, the lipid-rich, oily substance produced by the sebaceous glands (Albone, 1984: 45–46, 60–64), acts as a controlled-release substance that delays the evaporation rate of volatile constituents (Regnier and Goodwin, 1977). Other long-chain compounds, *e.g.* squalene that is present in the secretions of many species, may also act as controlled-release substances. Squalene is present in high concentrations in the lipid fraction of the marking fluid of the male Bengal tiger and it has been shown that the lipid fraction of the marking fluid displays a high affinity for the volatile constituents present in its marking fluid (Burger *et al.*, 2008). Lanolin, present on the wool of sheep, could also play an essential role as controlled-release carrier of the semiochemicals used for kin recognition in this species.

In addition, the spatial range in which the signal is active is also important. The range can be increased by increasing the amount of material emitted, however this is energetically unfavourable for the animal. By increasing the surface area available for signal evaporation the amount of material need not be increased. It is very common for mammals to increase the surface area by releasing glandular secretions onto hair rather than directly into the environment (Alberts, 1992).

The informational content of the semiochemical message used for kin recognition should be such that the maximum amount of information is transmitted. This is made possible by increasing the number of chemical constituents released. Alberts (1992) found that a trend exists for recognition signals to contain a larger number of compounds, on average, than those signals used in sexual attraction or alarm-threat contexts. This probably reflects the higher molecular complexity necessary to provide information about the signaller's identity. An energetically favourable method of increasing a signal's information content is to create complex odours composed of volatile constituents through anaerobic activity (Albone *et al.*, 1977). The anal pockets of the Indian mongoose contain six volatile carboxylic acids that are present in different relative concentrations in different individuals and allow for recognition. Although individual mongooses produce similar substrates, it

---

---

seems that each mongoose is colonised by slightly different bacterial flora that produce a unique odour (Gorman, 1976).

### 1.2.3 Proteins involved in kin recognition

Mammalian semiochemical secretions and excretions are mostly complex mixtures, containing volatile molecules and, in some cases, also proteins. Growing evidence in the literature suggests that both proteins and small molecules may function as mammalian pheromones (Dulac and Torello, 2003). Proteins can either act as carrier molecules, known as pheromone-binding proteins (PBPs), that are involved in uptake, transport and delivery of odour molecules to chemosensory receptor neurons (Leal *et al.*, 2009), or the proteins can act on their own as semiochemicals, as do certain major urinary proteins (MUPs) and major histocompatibility complex (MHC) molecules.

PBPs are members of a lipocalin family of ligand-binding proteins found in secretions, saliva and urine. The  $\beta$ -barrel structure of the lipocalin with an enclosed ligand-binding calyx makes it possible for these proteins to bind and transport small volatile chemosignals (Flower, 1996). Rodents make use of these lipocalin proteins to convey information about sex, strain and species (Brennan and Kendrick, 2006). MUPs present in large quantities in mice urine, belong to the lipocalin protein family and they perform a number of functions (Touhara, 2008). The MUPs found in the urine of mature male mice promote aggressive behaviour between males (Chamero *et al.*, 2007), they convey information concerning individual identity (Beynon and Hurst, 2004), they play an important role in the mechanism of individual recognition (Hurst *et al.*, 2001), and the choice of mates appears to be influenced by MUP heterozygosity (Thom *et al.*, 2008). The lipocalin found in hamster vaginal secretion, aphrodisin, is also used in chemical communication (Henzel *et al.*, 1988). This protein selectively binds ligands, and the protein and ligand complex is necessary for pheromonal activity (Singer and Macrides, 1993).

MHCs belong to a large and highly polymorphic family of genes involved in self and non-self recognition by the immune system on a cellular level as a defence against invading

---

---

pathogens. MHCs can also determine individual identity at the behavioural level. Yamazaki *et al.* (1990) showed that MHC genotype influences the urine odour of mice, enabling them to discriminate between urine from different individuals in a Y maze (Brennan and Kendrick, 2006). Furthermore, the MHC peptide from unfamiliar male mice has been shown to induce a pheromone-based pregnancy block, known as the Bruce effect (Leinders-Zufall *et al.*, 2004).

### **1.3 The Domestic Sheep, *Ovis aries***

#### **1.3.1 Background**

Sheep (*Ovis aries*) have been domesticated for about 11 000 to 12 000 years and are widely distributed throughout the world, having adapted to many different climatic conditions. In the 21st century, sheep breeding is still very important to the economies of several countries. China is the largest producer of sheep products, followed by Australia and New Zealand in the southern hemisphere (Lynch *et al.*, 1992: 1–2). Sheep have also been the subject of considerable research and they are regarded as one of the most studied non-human and non-rodent species (Adams and McKinley, 1995).

#### **1.3.2 Maternal behaviour and lamb survival**

All studies of lamb survival and maternal behaviour emphasise the importance of the lamb's first few days of life. The most important conditions for lamb survival are the ability of the ewe to provide sufficient milk for her offspring and the establishment of the selective bond between the ewe and her offspring. Many lambs die within the first few days after birth, indicating that at least one of the above-mentioned prerequisites has not been met. Disease plays a negligible role in lamb losses, especially during the first days after birth. A ewe suffering from severe under-nutrition would be able to provide enough milk for lamb survival, even if the amount of milk provided is not sufficient to ensure an optimal lamb growth rate. Apparently, the common factor that characterises most post-partum lamb deaths is the disturbance or the prevention of the bond formation between ewes and their offspring (Lindsay, 1988: 32).

---

---

Ewes are generally not interested in lambs; they only show interest in them for a short period around the time of parturition. During this period ewes are known to steal other ewes' lambs. This interest in lambs and the onset of maternal behaviour needs to be enforced by contact with an active lamb within 4 to 6 hours after birth; if this fails to happen, maternal interest fades and the ewe will not accept any lamb. Once the bond has been formed between ewe and lamb they can be separated for relatively long periods without disruption of the bond (Lindsay, 1988: 32).

The breed of the ewe (Dwyer and Lawrence, 1998), the nutritional status of the ewe (Putu *et al.*, 1988), the ewe's temperament, her previous maternal experience and, to some extent, the behaviour of her lamb (Dwyer, 2008) are the main factors that may influence maternal behaviour and the formation of the bond between a ewe and her lamb. However, the maternal selectivity and ewe–lamb bond depends very strongly on the individual odour of the offspring, since a selective bond between a ewe and her own lamb does not develop in ewes that were made anosmic before parturition (Lévy *et al.*, 2004).

### 1.3.3 The olfactory system and offspring recognition in sheep

The imperative role of olfaction in offspring recognition has been extensively studied in numerous species, especially in sheep. Because sheep are seasonal breeders, a large number of ewes give birth in a short time period, which makes it crucial for each ewe to recognise her lamb in order to provide selective rearing for her offspring. Initially the ewe relies mainly on olfactory cues to recognise her lamb. Although auditory and visual cues are used once the maternal bond has been established, ewes always appear to rely on olfactory cues as the final assurance before allowing lambs at the udder (Lindsay, 1988: 33).

When young are not a priority for ewes, *i.e.* when the ewes are not in parturition and not lactating, olfactory cues play an inhibitory role in maternal responsiveness, leading to the ewes displaying indifferent or hostile behaviour towards neonatal lambs (Dwyer, 2008). However, during parturition, odours produced by, or associated with, the lambs become a

---

---

powerful stimulus and neural structures such as the olfactory bulb undergo extensive changes when exposed to these odours (Kendrick *et al.*, 1992).

There is no functional specificity of the MOS or the AOS in the development of maternal behaviour. However, it appears that only the MOS is implicated during ewe–lamb recognition (Lévy *et al.*, 2004). When the MOSs of ewes are rendered inefficient for olfactory detection by bullectomy (Baldwin and Shillito, 1974), sectioning of the olfactory nerves (Morgan *et al.*, 1975), or irrigating the olfactory mucosa with zinc sulphate solution (Lévy *et al.*, 1995; Poindron, 1976), the ewes cannot recognise their offspring and accept any lamb as well as their own (Lévy *et al.*, 2004). Lévy *et al.* (1995) established that the AOS is not involved in lamb recognition because severing the nerves of the AOS did not prevent ewes from responding to amniotic fluid or prevent the ewes from displaying selectivity towards their own lambs. The possible role of the AOS in individual recognition can, however, not be excluded since the results obtained by Booth (2006) showed that the VNO, and not the AOS, is necessary for ewes to recognise their offspring.

#### 1.3.4 Amniotic fluid and ewe–lamb bond formation

The onset of maternal behaviour in the ewe is characterised by a strong attraction to amniotic fluids (Lévy *et al.*, 1983). Ewes in gestation and in their oestrous cycle are repelled by amniotic fluid, but for a few hours after parturition they become extremely attracted to birth fluids. Parturient ewes prefer eating food contaminated with amniotic fluid to non-contaminated food (Lévy *et al.*, 1983; Lindsay, 1988: p.32) and ewes are more attracted to a model lamb smeared with amniotic fluid than to one without (Vince *et al.*, 1985). The attraction to amniotic fluid results in licking and grooming the lamb, low-pitched bleating and the acceptance of the lamb at the udder. These behaviours are directed towards the formation of the exclusive olfactory attachment between the ewe and lamb and rely on the ewe's ability to memorise the individual olfactory cues from her offspring (Lévy *et al.*, 2004).

Lévy and Poindron (1984) treated lambs that were rejected by their ewes with amniotic fluid and this treatment led to an increase in acceptance rate of the lambs.

---

---

However, the origin of the amniotic fluid had no effect on maternal acceptance, indicating that amniotic fluid contains cues for general attractiveness rather than for individual recognition (Lévy *et al.*, 2004).

### 1.3.5 Lamb fostering using odour manipulation

The adoption of alien lambs (add-on fostering) has been attempted by making use of either artificial odorants to establish a common odour for own and alien lambs, or by transferring the odour of the ewe's own lamb to the alien lamb, or by making use of a combination of these two techniques. Inhibiting the access of the ewe to the odour of the alien lamb has also been explored.

Alexander *et al.* (1987) used neatsfoot oil to aid fostering by masking the odour of alien lambs and to establish a common odour between own and alien lambs with success rates varying from 40% to 70%. Furthermore, many ewes required prolonged restraint before reaccepting their own lambs after separation for periods varying between 5 and 26 hours. Other artificial odorants used by Alexander *et al.* (1989a) in an attempt to facilitate adoption of alien-lambs were the non-polar substances white soft paraffin and liquid paraffin and the polar substances polyethylene glycol, glycerol, silicone oil and diisooctylphthalate. Adoption was not achieved, but maternal interest was increased by coating alien lambs with the apolar substances. The more polar substances elicited no interest. The ewes also showed no interest in alien lambs that were washed before they were coated with the apolar substances. The bond between the ewes and their own lambs was not affected by coating the lambs with either polar or nonpolar substances.

Odour transfer from lamb to alien lamb has been carried out in a number of different ways. Fostering alien lambs by odour transfer using nylon stockinettes (Price *et al.*, 1984) has been achieved by fitting lambs with the stockinettes for 48 to 72 hours and fitting alien lambs with these stockinettes turned inside out. Fostering attempts with alien lambs immediately after fitment of the stockinettes resulted in a low acceptance rate (38%), although acceptance rates of alien lambs after 36 hours of testing increased considerably

---

---

(84%). Alexander *et al.* (1989b) experimented with three odour transfer techniques. First, the odour of lambs was transferred by coating lambs with soft white paraffin, scraping it off and applying the paraffin to the wool of the alien lambs. Second, lambs were washed with water containing a detergent and the recovered water was used to coat the alien lamb. Third, hessian coats worn for *ca.* 24 hours by lambs were fitted to alien lambs; the lambs were tested immediately and again after 2 to 3 days of confinement. Low acceptance rates of 30% to 60% were initially obtained with all of these techniques. However, the acceptance rates improved considerably to a maximum of 93% after a few days of confinement.

Alexander *et al.* (1989b) have also explored inhibiting the ewes' access to the odour of alien lambs. Ewes were fitted with modified dog muzzles containing wool of their own lambs (*ca.* 2 g). Results of initial acceptance tests immediately following the treatment were low (30%) and also after 2 to 3 days of confinement (40%). Apparently, the fostering of alien lambs was facilitated by this treatment only as long as the wool was fresh. Frequent replacement of the wool was impractical due to the limited amount of wool on newborn lambs.

Price *et al.* (2003) facilitated adoption of alien lambs by making use of a combination of two adoption techniques. Neatsfoot oil was used to establish a common odour for the ewes' own and alien lambs. Stockinette jackets were used to transfer the odour from the ewes' own lambs to the alien lambs. An 85% success acceptance rate was obtained.

It is clear that the transfer of the odour of a lamb to an alien lamb is more effective than creating a common odour between own and alien lamb. The presence of lamb odour is also more important than the absence of the odour of the alien lamb because no attempts were made during the odour transfer experiments to eliminate the alien lamb's odour.

### 1.3.6 The use of semiochemicals in sheep husbandry

Synthetic semiochemicals are mainly used during reproductive processes in sheep husbandry. The "ram effect" describes the process by which ewes are stimulated to ovulate

---

---

when rams are introduced into a ewe flock. This effect is widely used in Australia to synchronise lambing in large flocks of sheep (Lindsay *et al.*, 1984). Radford and Watson (1957) suggested that semiochemicals might be involved since physical or visual contact is not required to induce oestrus in ewes and anosmic ewes failed to respond to the “ram effect”. Knight and Lynch (1980) and Izard (1983) showed that the semiochemicals present in the wool, wax and urine of a ram are sufficient to stimulate ewes to ovulate.

Currently, synthetic semiochemicals are not available to address the problems associated with the disruption of ewe–lamb bond formation.

## **1.4 Motivation and Aims of the Study**

The production of animal products makes a substantial contribution to South Africa’s economy. However, there are a number of factors that impact upon the productivity of this economic sector. The most important of these are low rainfall and the marginal soil quality in some areas where sheep and cattle farming are practiced (factors over which farmers have very little control). It is therefore important to limit stock losses as far as possible. As far as sheep farming is concerned, the Merino is the preferred race in South Africa. Ewes with twins often do not produce enough milk to feed both lambs, or ewes reject their lambs for unknown reasons. This is a well-known phenomenon in all sheep races, and sheep farmers and scientists have examined various methods of making alien lambs acceptable to foster ewes. The survival rate of offspring could possibly be substantially increased if a more reliable method could be devised to prevent ewes from rejecting their own lambs or if ewes with an ample supply of milk could be induced to accept lambs other than their own.

A time span of four weeks from the birth of the lamb is critical to the survival of lambs. During this time some ewes and 15% of lambs will die. One of the factors that plays an important role in the survival of lambs is the bond that forms between ewe and lamb within a few hours after the birth of the lamb. As explained above, there is strong evidence that the ewe–lamb bond formation has a pheromonal basis, that is, the ewe can recognise her



---

---

own lamb by its familiar smell. If a better understanding of the semiochemicals involved in this communication between ewe and offspring could be obtained it might be possible to develop a method by which ewes can be coaxed into adopting foster lambs.

According to information obtained from the websites of The Döhne Merino Sheep Breeders Society of South Africa and The Australian Döhne Breeders Association, the Döhne Merino is a synthetic, dual-purpose Merino developed by the South African Department of Agriculture using merino ewes and German Mutton Merino sires. The progeny were interbred and selected for high fertility, rapid lamb growth rate, and fine Merino wool under commercial rangeland conditions. The breeding programme was initiated in 1939 and the Breed Society was formed in 1966. Since 1970 selection has been carried out with the aid of performance and progeny testing and comprehensive production records. All recorded animals are maintained in a computerised flock-recording scheme (Olivier, 2003). The Döhne Merino is one of the leading wool-producing breeds in South Africa.

The main aim of this study was to investigate the semiochemical communication involved in neonatal offspring recognition in sheep. It is known that Döhne Merino ewes bear a high percentage of twins and it was argued that by comparing the chemical composition of the odour of one lamb of a twin with that of the other twin, and by comparing the odour of this twin with those of other lambs, it should be possible to establish which constituents of the odour of lambs play an essential role in the semiochemical communication between ewe and lamb. The research was envisaged to include an investigation of the role of the volatile constituents of the amniotic fluid as well as the volatile constituents of the wool of neonatal lambs in offspring recognition in the Döhne Merino sheep. By investigating all of these aspects it was hoped to gain a better understanding of the mechanisms involved in the recognition process. This understanding could lead to the development of methods to facilitate fostering of alien lambs and could contribute to economic viability of one of the important economic sectors of the country.

---

---

## References

Adams, D. and McKinley, M., “The Sheep”, *ANZCCART News* **8**, No. 2 (1995).  
Revised by Colditz, I. and Dart, C., *ANZCCART Fact Sheet*, University of Adelaide,  
**A9** (2009).

Alberts, A. C., “Constraints on the design of chemical communication systems in terrestrial vertebrates”, *Am. Nat.* **139**: S62–S89 (1992).

Albone, E. S., “Mammalian Semiochemistry. The Investigation of Chemical Signals Between Mammals”, John Wiley & Sons Ltd., New York (1984).

Albone, E. S., Gosden, P. E. and Ware, G. C., “Bacteria as a source of chemical signals in mammals” in “Chemical Signals in Vertebrates”, Vol. 1, Müller-Schwarze, D. and Mozell, M. M. (eds.), Plenum, New York, pp. 35–43 (1977).

Alexander, G., Goodrich, B. S., Stevens, D. and Bradley, L. R., “Maternal interest in lambs smeared with polar and nonpolar substances”, *Austr. J. Exp. Agric.* **29**: 513–515 (1989a).

Alexander, G., Stevens, D. and Bradley, L. R., “Fostering in sheep: experiences with the use of neatsfoot oil”, *Austr. J. Exp. Agric.* **27**: 771–778 (1987).

Alexander, G., Stevens, D. and Bradley, L. R., “Fostering in sheep: an exploratory comparison of several approaches”, *Austr. J. Exp. Agric.* **29**: 509–512 (1989b).

Baldwin, B. A. and Shillito, E. E., “The effects of ablation of the olfactory bulbs on parturition and maternal behaviour in Soay sheep”, *Anim. Behav.* **22**: 220–223 (1974).

Beynon, R. J. and Hurst, J. L., “Urinary proteins and the modulation of chemical scents in mice and rats”, *Peptides* **25**: 1553–1563 (2004).

---

---

Booth, K. K., “The significance of the vomeronasal organ for offspring recognition in sheep”, *Small Ruminant Res.* **62**: 39–41 (2006).

Bradbury, J. W. and Vehrencamp, S. L., “Principles of Animal Communication”, Sinauer Associates, Inc. Massachusetts (1998).

Brennan, P. A. and Kendrick, K. M., “Mammalian social odours: attraction and individual recognition”, *Phil. Trans. R. Soc. B* **361**: 2061–2078 (2006).

Brennan, P. A. and Keverne, E. B., “Something in the air? New insights into mammalian pheromones”, *Curr. Biol.* **14**: R81–R89 (2004).

Burger, B. V., “Mammalian Semiochemicals” in “The Chemistry of Pheromones and Other Semiochemicals II”, Schulz, S. (ed.), Topics in Current Chemistry, Springer-Verlag, Berlin, **240**: 231–278 (2005b).

Burger, B.V., Viviers, M. Z., Bekker, J. P. I., Le Roux, M., Fish, N., Fourie, W.B. and Weibchen, G., “Chemical characterization of territorial marking fluid of male Bengal tiger, *Panthera tigris*”, *J. Chem. Ecol.* **34**: 659–671 (2008).

Chamero, P, Marton, T. F., Logan, D. W., Flanagan, K., Cruz, J. R., Saghatelian, A., Cravatt, B. F. and Stowers, L., “Identification of protein pheromones that promote aggressive behaviour”, *Nature* **450**: 899–902 (2007).

Døving, K. B. and Trotier, D., “Structure and function of the vomeronasal organ”, *J. Exp. Biol.* **201**: 2913–2925 (1998).

Dulac, C. and Torello, A. T., “Molecular detection of pheromone signals in mammals: from genes to behaviour”, *Nat. Rev. Neurosci.* **4**: 551–562 (2003).

---

---

Dwyer, C. M., “Genetic and physiological determinants of maternal behaviour and lamb survival: implications for low-input sheep management”, *J. Anim. Sci.* **86**: E246–E258 (2008).

Dwyer, C. M. and Lawrence, A. B., “Variability in the expression of maternal behaviour in primiparous sheep: effects of genotype and litter size”, *Appl. Anim. Behav. Sci.* **58**: 311–330 (1998).

Flower, D. R., “The lipocalin protein family: structure and function”, *Biochem. J.* **318**: 1–14 (1996).

Gorman, M. L., “A mechanism for individual recognition by odour in *Herpestes auro punctatus* (Carnivora: Viverridae)”, *Anim. Behav.* **24**: 141–145 (1976).

Henzel, W. J., Rodriguez, H., Singer, A. G., Stults, J. T., Macrides, F., Agosta, W. C. and Niall, H., “The primary structure of aphrodisin”, *J. Biol. Chem.* **263**: 16682–16687 (1988).

Hepper, P. G. and Cleland, J., “Developmental aspects of kin recognition”, *Genetica* **104**: 199–205 (1999).

Hurst, J. L., Payne, C. E., Nevison, C. M., Marie, A. D., Humphries, R. E., Robertson, D. H., Cavaggioni, A. and Beynon, R. J., “Individual recognition in mice mediated by major urinary proteins”, *Nature* **414**: 631–634 (2001).

Izard, M. K., “Pheromones and reproduction in domestic animals” in Vandenberg, J.G. (Ed.), “Pheromones and Reproduction in Mammals”, Academic Press, New York, pp. 253–285 (1983). Cited in Rekwot, P. I., Ogwu, D., Oyedipe, E. O. and Sekoni, V. O., “The role of pheromones in biostimulation in animal reproduction”, *Anim. Reprod. Sci.* **65**: 157–170 (2001).

---

---

Johnston, R. E., “Pheromones, the vomeronasal system, and communication: from hormonal responses to individual recognition”, *Ann. N. Y. Acad. Sci.* **855**: 333–348 (1998).

Karlson P. and Lüscher, M., “‘Pheromones’: a new term for a class of biologically active substances”, *Nature* **183**: 55–56 (1959).

Keller, M., Baum, M. J., Brock, O., Brennan, P. A. and Bakker, J., “The main and the accessory olfactory systems interact in the control of mate recognition and sexual behaviour”, *Behav. Brain Res.* **200**: 268–276 (2009).

Kendrick, K. M., Lévy, F. and Keverne, E. B., “Changes in the sensory processing of olfactory signals induced by birth in sheep”, *Science* **256**: 833–836 (1992).

Knight, T. W., and Lynch, P. R., “Source of ram pheromones that stimulate ovulation in the ewe”, *Anim. Reprod. Sci.* **3**: 133–136 (1980).

Law J. H. and Regnier, F. E., “Pheromones”, *Ann. Rev. Biochem.* **40**: 533–548 (1971).

Leal, W. S., Ishida, Y., Pelletier, J., Xu, W., Rayo, J., Xu, X. and Ames, J. B., “Olfactory proteins mediating chemical communication in the navel orangeworm moth, *Amyelois transitella*”, *PLoS ONE* **4**: e7235 (2009).

Leinders-Zufall, T., Brennan, P., Widmayer, P., Chandramani, P. S., Maul-Pavicic, A., Jager, M., Li, X-H., Breer, H., Zufall, F. and Boehm, T., “MHC class I peptides as chemosensory signals in the vomeronasal organ”, *Science* **306**: 1033–1037 (2004).

Lévy, F., Keller, M. and Poindron, P., “Olfactory regulation of maternal behavior in mammals”, *Horm. Behav.* **46**: 284–302 (2004).

---

---

Lévy, F., Locatelli, A., Piketty, V., Tillet, Y. and Poindron, P., “Involvement of the main but not the accessory olfactory system in maternal behaviour of primiparous and multiparous ewes”, *Physiol. Behav.* **57**: 97–104 (1995).

Lévy, F. and Poindron, P., “Influence du liquide amniotique sur la manifestation du comportement maternel chez la brebis parturiente (The influence of amniotic fluid on maternal behavior in parturient sheep)”, *Biol. Behav.* **9**: 65–88 (1984). Cited in Lévy, F., Keller, M. and Poindron, P., “Olfactory regulation of maternal behavior in mammals”, *Horm. Behav.* **46**: 284–302 (2004).

Lévy, F. and Poindron, P., “The importance of amniotic fluids for the establishment of maternal behavior in experienced and inexperienced ewes”, *Anim. Behav.* **35**: 1188–1192 (1987).

Lévy, F., Poindron, P. and Le Neindre, P., “Attraction and repulsion by amniotic fluids and their olfactory control in the ewe around parturition”, *Physiol. Behav.* **31**: 687–692 (1983). Cited in Numan, M. and Insel, T. R., “The Neurobiology of Parental Behavior”, Springer, New York (2003) p. 120.

Lévy, F., Porter, R. H., Kendrick, K. M., Keverne, E. B and Romeyer, A., “Physiological, sensory, and experiential factors of parental care in sheep”, *Adv. Stud. Behav.* **25**: 385–422 (1996). Cited in Lévy, F., Keller, M. and Poindron, P., “Olfactory regulation of maternal behavior in mammals”, *Horm. Behav.* **46**: 284–302 (2004).

Lindsay, D., “Breeding the Flock: Modern Research and Reproduction in Sheep”, Inkata Press, Melbourne (1988).

Lindsay, D. R., Gray, S. J., Oldham, C. M. and Pearce, D. T., “The single injection of progesterone” in “Proceedings of the Australian Society of Animal Production”, Vol. 15, pp. 159–161 (1984). Cited in Rekwot, P. I., Ogwu, D., Oyedipe, E. O. and

---

---

Sekoni, V. O., “The role of pheromones in biostimulation in animal reproduction”, *Anim. Reprod. Sci.* **65**: 157–170 (2001).

Luo, M. M., Fee, M. S. and Katz, L. C., “Encoding pheromonal signals in the accessory olfactory bulb of behaving mice”, *Science* **299**: 1196–1201 (2003).

Lynch, J. J., Hinch, G. N. and Adams, D. B., “The Behaviour of Sheep. Biological Principles and Implications for Production”, C·A·B International and CSIRO Australia, Melbourne (1992).

Morgan, P. D., Boundy, C. A., Arnold, G. W. and Lindsay, D. R., “The roles played by the senses of the ewe in the location and recognition of lambs”, *Appl. Anim. Ethol.* **1**: 139–150 (1975).

Olivier, J. J., Shepherd Computerised flock-recording program, ARC Animal Improvement Institute, RSA (2003).

Poindron, P., “Mother–young relationships in intact or anosmic ewes at the time of suckling”, *Biol. Behav.* **2**: 161–177 (1976). Cited in Lévy, F., Keller, M. and Poindron, P., “Olfactory regulation of maternal behavior in mammals”, *Horm. Behav.* **46**: 284–302 (2004).

Poindron, P. and Le Neindre, P., “Endocrine and sensory regulation of maternal behavior in the ewe”, *Adv. Study Behav.* **11**: 75–119 (1980). Cited in Lévy, F., Keller, M. and Poindron, P., “Olfactory regulation of maternal behavior in mammals”, *Horm. Behav.* **46**: 284–302 (2004).

Price, E. O., Dally, M. R. and Hernandez, L., “A note on the use of odor manipulation to facilitate the adoption of alien lambs by ewes bearing twins”, *Appl. Anim. Behav. Sci.* **81**: 127–131 (2003).

---

---

Price, E. O., Dunn, G. C., Talbot, J. A. and Dally, M. R., “Fostering lambs by odor transfer: the substitution experiment”, *J. Anim. Sci.* **59**: 301–307 (1984).

Putu, I. G., Poindron, P. and Lindsay, L. R., “Early separation of ewes from the birth site increases lamb separations and mortality”, *Proc. Aust. Soc. Anim. Prod.* **17**: 298–301 (1988). ). Cited in Dwyer, C. M., “Genetic and physiological determinants of maternal behavior and lamb survival: implications for low-input sheep management”, *J. Anim. Sci.* **86**: E246–E258 (2008).

Radford, H. M. and Watson, R. H., “Influence of rams on ovarian activity and oestrus in Merino ewes in the spring and early summer”, *Aust. J. Agric. Sci.* **8**: 260 (1957).

Regnier, F. E. and Goodwin, M., “On the chemical and environmental modulation of pheromone release from vertebrate scent marks” in “Chemical signals in vertebrates”, Vol. 1, Müller-Schwarze, D. and Mozell, M. M. (eds.), Plenum, New York, pp. 115–133 (1977).

Rekwot, P. I., Ogwu, D., Oyedipe, E. O. and Sekoni, V. O., “The role of pheromones and biostimulation in animal reproduction”, *Anim. Reprod. Sci.* **65**: 157–170 (2001).

Restrepo, D., Arellano, J., Oliva, A. M., Schaefer, M. L. and Lin, W., “Emerging views on the distinct but related roles of the main and accessory olfactory systems in responsiveness to chemosensory signals in mice”, *Horm. Behav.* **46**: 247–256 (2004).

Schaefer, M. L., Yamazaki, K., Osada, K., Restrepo, D. and Beauchamp, G. K., “Olfactory fingerprints for major histocompatibility complex-determined body odors II: relationship among odor maps, genetics, odor composition, and behaviour”, *J. Neurosci.* **22**: 9513–9521 (2002).

Serizawa, S., Ishii, T., Nakatani, H., Tsuboi, A., Nagawa, F., Asano, M., Sudo, K., Sakagami, J., Sakano, H., Ijiri, T., Matsuda, Y., Suzuki, M., Yamamori, T., Iwakura,



---

---

Y. and Sakano, H., “Mutually exclusive expression of odorant receptor transgenes” *Nat. Neurosci.* **3**:687–693 (2000).

Shorey, H. H., "Animal Communication by Pheromones", Academic Press, New York (1976).

Singer, A. G., Beauchamp, G. K. and Yamazaki, K., “Volatile signals of the major histocompatibility complex in male mouse urine”, *Proc. Natl Acad. Sci. USA* **94**: 2210–2214 (1997).

Singer, A. G. and Macrides, F., “Composition of an aphrodisiac pheromone”, *Chem. Senses* **18**: 630 (1993). Cited in Henzel, W. J., Rodriguez, H., Singer, A. G., Stults, J. T., Macrides, F., Agosta, W. C. and Niall, H., “The primary structure of aphrodisin”, *J. Biol. Chem.* **263**: 16682–16687 (1988).

Song H-G., Kwon, J. Y., Han, H. S., Bae, Y-C. and Moon, C., “First contact to odors: our current knowledge about odorant receptors”, *Sensors* **8**: 6303–6320 (2008).

Thom, M. D., Stockley, P., Jury, F., Ollier, W. E. R., Beynon, R. J. and Hurst, J. L., “The direct assessment of genetic heterozygosity through scent in the mouse”, *Curr. Biol.* **18**: 619–623 (2008).

Touhara, K., “Sexual communication via peptide and protein pheromones”, *Curr. Opin. Pharmacol.* **8**: 759–764 (2008).

Trinh, K. and Storm, D. R., "Vomer nasal organ detects odorants in absence of signaling through main olfactory epithelium", *Nat. Neurosci.* **6**: 519–525 (2003).

Vince, M. A., Lynch, J. J., Mottershead, B., Green, G. and Elwin, R., “Sensory factors involved in immediately postnatal ewe/lamb bonding”, *Behaviour* **94**: 60–84 (1985).

Wilson, E. O., "Pheromones", *Sci. Am.* **208**: 100–114 (1963).

Wilson, E. O., "Sociobiology", Belknap Press, Harvard, Massachusetts (1975). Cited in Wyatt, T. D., "Pheromones and Animal Behaviour. Communication by Smell and Taste", Cambridge University Press, Cambridge (2003) p.3.

Wyatt, T. D., "Pheromones and Animal Behaviour. Communication by Smell and Taste", Cambridge University Press, Cambridge (2003).

Wyatt, T. D., "Pheromones: convergence and contrasts in insects and vertebrates" in "Chemical Signals in Vertebrates", Vol. 10, Mason, R. T., LeMaster, M. P. and Müller-Schwarze, D. (eds.), Springer Science and Business Media, Inc., Oregon, pp. 7–20 (2005).

Yamazaki, K., Beauchamp, G. K., Imai, Y., Bard, J., Phelan, S. P., Thomas, L. and Boyse, E. A., "Odortypes determined by the major histocompatibility complex in germfree mice", *Proc. Natl. Acad. Sci. USA* **87**: 8413–8416 (1990).

# CHAPTER 2

## EXPERIMENTAL

### 2.1 General

All Pyrex (borosilicate) glassware used for the collection of samples was thoroughly washed with distilled water, heated in an annealing oven at 400 °C for at least 30 min to remove all traces of adsorbed organic material and then cooled immediately prior to use. Samples (1 ml) of the solvents dichloromethane (DCM) (Romil, Super Purity, Cambridge, UK) and *tert*-butyl methyl ether (TBME) (Sigma-Aldrich, anhydrous, 99.8%, St Louis, USA) were concentrated to 10 µl in a nitrogen atmosphere as described in § 2.3.3.1, and analysed for impurities. Both solvents were found to be sufficiently pure for the extraction of semiochemicals. Syringes were cleaned by flushing with DCM. The plungers were rinsed with DCM. The stainless steel spoon, tweezers and scissors used for the collection of amniotic fluid and wool samples from sheep were washed with 70% ethanol (Merck, Darmstadt, Germany) and dried prior to use.

### 2.2 Sample Collection and Preparation

A ewe flock of Döhne Merinos was available for the collection of samples and bioassays at Mariendahl, an experimental farm of Stellenbosch University, located 14 km outside Stellenbosch. Single- and twin-bearing ewes were housed in separate paddocks. After the birth of the lambs the single-bearing ewes with their lambs were moved to their own paddock and the twin-bearing ewes with their lambs were moved to lambing pens for 2 to 3 days, after which they were housed in their own paddock. For statistical purposes, sixteen of the one-hundred-and-sixty-five twin-bearing ewes were used as a sample group (9.69%).

Amniotic fluid was scraped off the body of each newborn lamb with a stainless steel spoon (Plate 2.1a) immediately after the ewe had given birth. Amniotic fluid samples (*ca.* 10–30 ml) were collected and stored in glass bottles with Teflon-lined screw caps. The

samples were kept on ice in the field and then stored at  $-20\text{ }^{\circ}\text{C}$  in the laboratory until analysed. Amniotic fluid was collected for protein analysis in 2 ml Eppendorf tubes (Plate 2.1b). Wool samples (*ca.* 200–400 mg) were collected from the foreheads of 32 lambs on the morning after they were born (day 1) and from five twins (10 lambs) on day 7 (Plate 2.1c). The wool samples were stored in glass bottles with Teflon-lined screw caps and stored at  $-20\text{ }^{\circ}\text{C}$  until analysed.

The processing of the amniotic fluid and the wool samples for gas chromatographic separation in conjunction with gas chromatographic-low resolution mass spectrometric (GC-MS) analysis is summarised in Figs. 2.1 and 2.2, respectively:

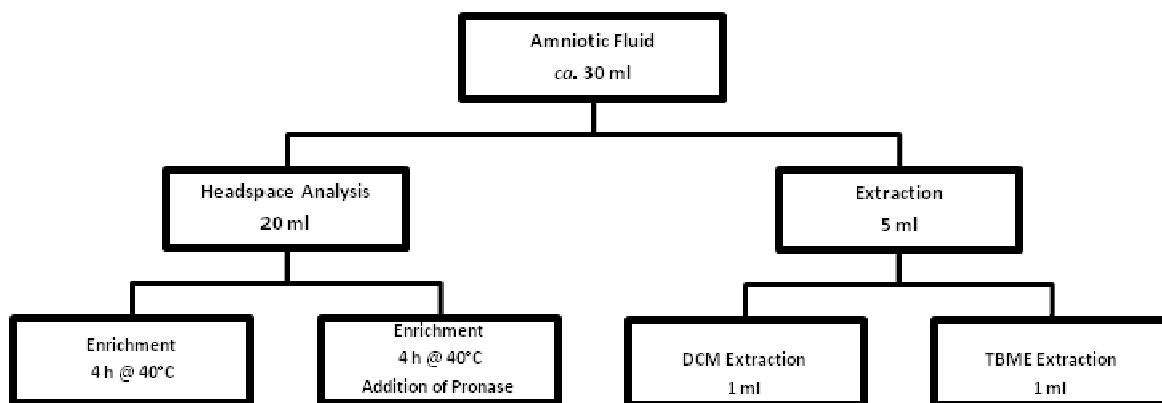


Fig. 2.1. Sample allocation of the amniotic fluid for GC-MS analysis.

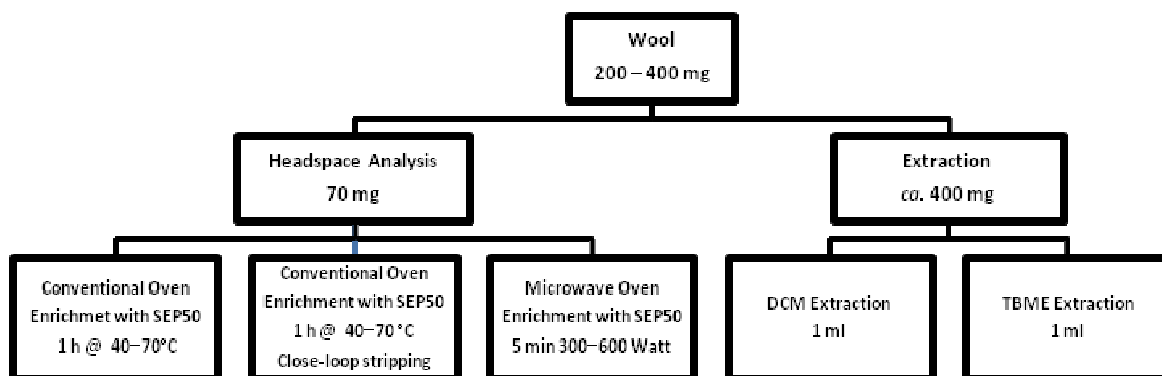


Fig. 2.2. Sample allocation of wool for GC-MS analysis.

## 2.3 Analytical Methods

### 2.3.1 Gas chromatographic analysis

Gas chromatographic (GC) analyses were carried out with a Carlo Erba HRGC gas chromatograph (Milan, Italy) with a flame ionisation detector (FID) and a Grob split/splitless sample inlet. Gas chromatographic data were acquired with a DELTA Chromatography Data System, Version 5.0 (Digital Solutions, Brisbane, Australia). The capillary columns used were manufactured by the Laboratory for Ecological Chemistry (LECUS, Stellenbosch University) and were provided with integrated retention gaps of 1 to 2 m. The following columns were used: column A [glass, 40 m  $\times$  0.25 mm i.d., coated with 0.25  $\mu$ m of PS-089-OH (DB-5 equivalent)], column B [glass, 40 m  $\times$  0.25 mm i.d., coated with 0.25  $\mu$ m of the polar stationary phase AT-1000 (FFAP equivalent)], enantioselective column C [glass, 30 m  $\times$  0.3 mm i.d., coated with 0.25  $\mu$ m of OV-1701-OH containing 10% heptakis(2,3-di-O-methyl-6-O-tert-butyldimethylsilyl)- $\beta$ -cyclodextrin] and enantioselective column D [glass, 30 m  $\times$  0.3 mm i.d., coated with 0.25  $\mu$ m of OV-1701-OH containing 10% heptakis(2,3-di-O-acetyl-6-O-tert-butyldimethylsilyl)- $\beta$ -cyclodextrin)]. Hydrogen was used as carrier gas at a linear velocity of 50 cm/s (column temperature 40  $^{\circ}$ C). The injector was operated at 220  $^{\circ}$ C and the FID at 280  $^{\circ}$ C. Samples were injected in the split mode (split ratio 1:10) at a column temperature below 30  $^{\circ}$ C. The column temperature was then ballistically increased to 40  $^{\circ}$ C, after which temperature programmes of 2  $^{\circ}$ C/min from 40  $^{\circ}$ C to 280  $^{\circ}$ C and 2  $^{\circ}$ C/min from 40  $^{\circ}$ C to 250  $^{\circ}$ C were used for columns A and B, respectively. The final temperature was kept isothermic for 20 min at either 250  $^{\circ}$ C or 280  $^{\circ}$ C. The enantioselective columns were programmed from 40  $^{\circ}$ C to 220  $^{\circ}$ C at 1 $^{\circ}$ C/min.

### 2.3.2 Gas chromatographic-mass spectrometric analysis

Gas chromatographic-low resolution electron impact mass spectrometric analyses (GC-LRMS) of material isolated from the amniotic fluid and wool samples were carried out on a Carlo Erba QMD 1000 GC-MS instrument (Milan, Italy). The GC parameters and capillary columns specified above were used in GC-MS analyses. Electron impact (EI) mass spectral data were acquired at 70 eV from  $m/z$  25 to 350. Helium was used as carrier gas at a

linear velocity of 28.6 cm/s (column temperature 40 °C). Inlet and interface temperatures were 220 °C and 250 °C, respectively. The ion source temperature was set at 180 °C and the pressure in the source housing was *ca.*  $2 \times 10^{-5}$  mm Hg at a column temperature of 40 °C, decreasing to *ca.*  $1 \times 10^{-5}$  mm Hg towards the end of the temperature programme. A scan rate of 0.9 s/scan and an interscan time of 0.1 s were used. This instrumentation was also used for retention time comparison of the volatile constituents of the amniotic fluid and wool samples<sup>1</sup> with authentic synthetic reference compounds.

High resolution mass spectrometric data were acquired on a Waters GCT Premier time-of-flight mass spectrometer (GC-TOF-HRMS) (Waters, Massachusetts, USA). The GC was fitted with capillary column E [30 m  $\times$  0.25 mm i.d., coated with 0.25  $\mu$ m of 5% phenyl-methylpolysiloxane) (DB-5ms, Agilent JW Scientific, Folsom, USA)]. Helium was used as carrier gas at 1 ml/min. An inlet temperature of 260 °C was used. Samples were introduced in the split mode (split ratio 1:5) at a column temperature below 30 °C. The column temperature was then ballistically increased to 40 °C, after which the column was programmed at 2 °C/min from 40 °C to 280 °C (isothermic for 5 min), followed by a 2 °C/min ramp to 300 °C (isothermic for 20 min). EI mass spectral data were acquired at 70 eV, scanning the mass range from  $m/z$  35 to 600 at a scan rate of 0.2 s/scan, with an interscan delay of 0.05 s. The source temperature was 180 °C and perfluoro-tri-n-butylamine was used as mass reference.

### 2.3.3 Nuclear magnetic resonance spectrometry

<sup>1</sup>H and <sup>13</sup>C nuclear magnetic resonance spectrometry (NMR) spectra were recorded in CDCl<sub>3</sub> on Varian VnmrS 300, Unity INOVA 400 and UNITY INOVA 600 NMR instruments (Varian, Palo Alto, USA). Chemical shifts are given in parts per million ( $\delta$ ) relative to chloroform (7.26 and 77.04 ppm for <sup>1</sup>H and <sup>13</sup>C NMR, respectively).

---

<sup>1</sup> For the sake of simplicity the VOCs present in the collected amniotic fluid and wool samples will be referred to as amniotic fluid volatiles and wool volatiles, respectively.

## 2.3.4 Sampling methods

### 2.3.4.1 Conventional sample extraction

DCM and TBME were considered as possible solvents for the extraction of the amniotic fluid and wool volatiles from the collected samples. TBME was found to be the more effective solvent (it extracted more volatile material) and the amniotic fluid and the wool volatiles were therefore extracted with small volumes (1 ml) of TBME. Octane (0.94  $\mu\text{g/ml}$  TBME) was used as internal standard in quantitative analyses of the amniotic fluid volatiles. Methyl hexanoate (14.8  $\mu\text{g/ml}$ ) was used as internal standard in analyses of the wool volatiles. Alternatively, synthetic analogues of the VOCs identified in this study were used as external standards.

On extracting the organic volatiles from the amniotic fluid with these solvents, a gel was formed. This was ascribed to the denaturing of proteins in the fluid. Separating the phases was facilitated by centrifugation for 1 min at 3000 rpm, freezing the material at  $-20$  °C and pouring off the supernatant solvent layer into a 2-ml vial. The extract was concentrated in a nitrogen atmosphere by placing the vial in a 100-ml beaker, covering the beaker with aluminium foil, and purging the solvent vapour from the beaker without directing the nitrogen flow into the vial. During the evaporation process, the extract was transferred to a smaller Reacti-Vial (Reiter *et al.*, 2003). The concentration process took about 15 h to yield a final volume of *ca.* 10  $\mu\text{l}$  of the concentrated extract. Aliquots (1  $\mu\text{l}$ ) of the amniotic fluid or wool extracts were subjected to GC-MS analysis using split and septum purge flows of 10 and 1 ml/min respectively.

### 2.3.4.2 Solid phase micro-extraction headspace sampling

The headspace volatiles of a sample of wool (70 mg) in a bottle (8 ml) were sampled by solid phase micro-extraction (SPME) using a 100  $\mu\text{m}$  PDMS fibre. The VOCs were enriched for 15 h at 50 °C in a conventional laboratory oven with air circulation. The sample was desorbed in the injector of the instrument 30 min at 220 °C.

### 2.3.4.3 Sample enrichment probe headspace sampling

A sample enrichment<sup>2</sup> probe (SEP) (MasChrom Analisetegniek, Stellenbosch, South Africa) was used to extract (enrich) the volatile organic material from the amniotic fluid and the wool samples for GC and GC-MS analyses.

A fresh sample of amniotic fluid (20 ml), selected for its clear appearance, was placed in a 50-ml glass bottle with a stainless steel insert that had the same thread as the injector cap of the GC (Burger *et al.*, 2006). The amniotic fluid was stirred at 40 °C with a glass-encapsulated magnetic follower (stirrer bar). A SEP50 (stalk, 130 x 0.70 mm) carrying a 50-mm sleeve of polydimethylsiloxane (PDMS) tubing (47 mg) near its tip (Plate 2.1d), was exposed to the headspace gas of the amniotic fluid for 4 h to enrich the organic volatiles from the amniotic fluid. The volatile compounds were desorbed at 220 °C in the GC-MS injector, and the SEP was left in the injector until completion of the analysis (Burger *et al.*, 2006).

The wool volatiles were sampled in a similar manner. Thus a SEP50 was exposed to the headspace of a sample of the wool (400 mg in a 50-ml glass bottle) at 40°C for a period of 2 h (Plate 2.1e). The volatile compounds were desorbed at 220 °C in the GC-MS injector.

Although sampling the volatiles from 70 mg of wool in an 8-ml bottle (Plate 2.1f) instead of from 400 mg in a 50-ml bottle (wool mass:bottle volume *ca.* 8.8 instead of 8.0) was not expected to bring about a large increase in SEP extraction efficiency, this adaptation did result in an appreciable increase in the sensitivity of the method.

In a further attempt to increase the efficiency of the SEP enrichment of the wool volatiles, an adapted version of a technique known as closed-loop stripping (Grob, 1973) was used. Using a lubricant-free pump (Metal Bellows Corp, Sharon, USA), air was circulated for 2 h over a sample of wool (70 mg) in a flow-through cell held at 40 °C, followed by using a smaller cell at 22 °C in which the PDMS rubber of a SEP50 was exposed to the headspace

---

<sup>2</sup> The recently introduced term “enrichment” is preferred for sampling or trapping techniques in which the constituents of a gas sample, or the headspace of solids or liquids, are concentrated in the sample preparation step prior to introduction of the sample into the injector of the analytical instrument.



---

---

gas of the wool. Only a slight increase in enrichment efficiency was obtained. This technique was subsequently rejected because the pump was contaminated by the compounds present in the wool to such an extent that the pump could not be cleaned by overnight pumping of purified air (activated charcoal). Even after circulating water, methanol, acetone, methanol and DCM (in this order) through the pump and purging the solvent vapours from the pump by overnight pumping of purified air, traces of the wool volatiles could still be detected when a blank analysis was carried out.

Exploratory experiments were conducted with a domestic microwave oven at power settings ranging from 300 W to 600 W, and using continuous heating (5 – 10 min) and pulsed heating (5 x 1 min at 600 W, with 5 min between pulses). Continuous heating for 5 min at 600 W proved more efficient for enrichment than pulsed heating did. Increasing the enrichment time combined with decreased power settings proved less efficient. The internal temperature of the domestic microwave oven operated at 600 W rose to *ca.* 145 °C. Oven temperatures were determined by using the melting points of selected crystalline compounds.

#### 2.3.4.4 Analysis of synthetic standards and retention time comparison

GC-MS analysis was used to monitor the progress of the laboratory syntheses of reference compounds and to confirm the identification of the compounds present in the amniotic fluid of the Döhne Merino ewes and the wool of their lambs by mass spectral and retention time comparisons with commercially available synthetic compounds, compounds that were available from previous research projects, or compounds that were synthesised during the present investigation. The synthetic compounds were made up in DCM, either individually or as mixtures containing the reference compounds in relative concentrations corresponding to those of the compounds identified in the amniotic fluid or wool. Kovats retention indices (Kovats, 1958) were determined only on apolar column A to substantiate the identification of amniotic fluid and wool volatiles.

## 2.4 Dimethyl Disulphide Derivatisation

The double bond positions of the unsaturated volatile organic constituents (VOCs) of the amniotic fluid and wool samples collected from ewes and lambs, respectively, were determined by dimethyl disulphide (DMDS) derivatisation and GC-MS analysis of the resulting derivates, according to Vincenti *et al.* (1987). An aliquot of the TBME extract of the amniotic fluid or the wool was concentrated to 5  $\mu\text{l}$  in a Reacti-Vial using a slow stream of purified nitrogen. The concentrated extract was redissolved in carbon disulphide (50  $\mu\text{l}$ ), and treated with an excess of iodine solution (5  $\mu\text{l}$  of a solution of 60 mg of  $\text{I}_2$  in 1 ml of TBME) and DMDS (50  $\mu\text{l}$ ). The Reacti-Vial was sealed and the reaction mixture heated in an oven at 60  $^{\circ}\text{C}$  for 40 h. The reaction was then quenched with a 5% aqueous sodium thiosulphate solution, and the aqueous layer separated from the organic layer by centrifuging the mixture at 3000 rpm. The organic layer was transferred to another Reacti-Vial and washed with water (2 x 20  $\mu\text{l}$ ), utilising centrifugation to facilitate separation of the phases. The extract was concentrated as described in § 2.3.3.1 and used for GC-MS analyses.

## 2.5 Determination of Absolute Configuration of Chiral Compounds

GC-MS analyses were carried out as described in § 2.3.2. Enantioselective columns C and D were used to separate the enantiomers of the chiral constituents present in the wool samples. The order in which the enantiomers of several of the chiral compounds identified in this study elute from columns coated with the same phases as columns C and D has previously been determined by Maas *et al.* (1994) and Burger *et al.* (2008).

## 2.6 Protein Identification

The protein content of the amniotic fluid collected from three pairs of twin lambs was estimated according to Bradford (1976), using serum albumin as standard, and found to be *ca.* 0.52  $\mu\text{g}/\mu\text{l}$ . The proteins were concentrated by precipitation, by adding an equal volume of 20% trichloroacetic acid (TCA) to the protein sample and incubating the mixture on ice for 30 min, followed by centrifugation at 13 000 rpm (16 000 g) for 15 min at 4  $^{\circ}\text{C}$ . The

supernatant was removed and cold acetone (300  $\mu$ l) was added. The sample centrifuged at 13 000 rpm (16 000 g) for 15 min at 4 °C. The supernatant was removed and the protein pellet dried.

The wool-associated proteins of two single lambs and one pair of twins, all of the same age, were identified. To remove the wool-associated proteins from the wool without denaturing the wool keratin proteins, wool of each lamb (2.5 g) was placed in a 150-ml Erlenmeyer flask and 0.5% sodium dodecyl sulphate (SDS) in 50 mM sodium phosphate pH 6 buffer (50 ml) was added and the mixture shaken for 2.5 h at 30 °C. The mixture was then placed in a 100-ml syringe, the aqueous phase separated from the wool and then centrifuged at 5000 g for 10 min. The aqueous phase was decanted from the dirt-containing pellet and an equal volume of 20% TCA was added. The mixture was stored overnight at -20 °C. After it had reached room temperature the aqueous phase was resuspended in 0.1 M phosphate pH 6 buffer and placed in a 15-cm length of high retention, seamless cellulose dialysis tubing (dialysis tubing: size 32 x 20 mm, MW 06205, D0530-100FT, Sigma-Aldrich) in a 10-l glass beaker and dialysed against 10 mM sodium phosphate buffer (pH 6) for 24 h at 4 °C. The protein concentration was found to be *ca.* 0.78  $\mu$ g/ $\mu$ l. The proteins were concentrated, precipitated and dried as described for the amniotic fluid proteins.

### 2.6.1 One-dimensional gel electrophoresis

Proteins were separated on 8% SDS-polyacrylamide (SDS-PAGE) gels according to their molecular weight, as described by Laemmli (1970). Gels had a total length of 74 mm and a thickness of 1 mm. The SDS-PAGE gels consisted of a running gel and a stacking gel:

*8% Running gel:* 0.375 M *tris*(hydroxymethyl)aminomethane (Tris-HCl) pH 8.8; 0.1% SDS (v/v); 8% (v/v) 30% acryl-bisacrylamide stock solution (Sigma); 0.75% ammonium persulphate (v/v) and 0.075% N,N,N',N'-tetramethylethylene-diamine (TEMED) (v/v).

*4% Stacking gel:* 0.125 M Tris-HCl pH 6.8; 0.1% SDS (v/v); 4% (v/v) 30% acryl-bisacrylamide stock solution (Sigma); 0.75% ammonium persulphate (v/v) and 0.075% TEMED (v/v).

A vertical slab gel was cast at room temperature by first preparing the running gel and allowing it to polymerise before overlaying it with the stacking gel. This was done on a Bio-Rad Mini Protean II (Bio-Rad, Hercules, California). Following precipitation, the crude protein pellets were resuspended in a reducing sample buffer (2 x: 0.5 M Tris-HCl pH 6.8; 10% SDS (w/v); 10% glycerol (v/v); 5%  $\beta$ -mercapto-ethanol (v/v) and 0.05% bromophenol blue (w/v)) and incubated at 65 °C for 3 min. The molecular weight markers (SDS-7B prestained SDS molecular weight marker [Sigma-Aldrich, St Louis, USA] was used for amniotic fluid proteins, and Fermentas PageRuler [Prestained Protein Ladder, #SM0671, Burlington, Canada] for the wool-associated proteins) were loaded as instructed by the supplier. Samples were separated in 1 x SDS-PAGE running buffer (5 x: 15 g/L Tris-HCl, 72 g/L glycine and 5 g/L SDS) at a constant potential of 150 V until the dye front had progressed to the bottom of the gel. Gels were stained for 30 min with coomassie blue R-250 staining solution (0.25% coomassie blue R-250 (w/v); 45% methanol; 10% glacial acetic acid) according to Sambrook and Russell (2001: A8.46–A8.47). Destaining was carried out in 3 to 5 changes of destaining solution (45% methanol, 10% glacial acetic acid). In Figs. 3.92 and 3.93 typical separations of amniotic fluid and wool proteins on SDS-PAGE gels are shown.

## 2.6.2 In-gel tryptic digestion

In-gel tryptic digestion was carried out according to the method of Shevchenko *et al.* (1996). The stained gel bands containing one or more proteins were cut from the SDS-PAGE gel and into smaller fragments (*ca.* 1 mm<sup>2</sup>), and placed in Eppendorf tubes. Acetonitrile (35  $\mu$ l) was added and the sample incubated for 10 min at room temperature. The liquid was removed and the gel pieces dried under vacuum with centrifugation (SpeedVac) for 10 min until complete dryness. The proteins were reduced by adding 10 mM dithiothreitol (DTT) in 100 mM NH<sub>4</sub>HCO<sub>3</sub> (150  $\mu$ l) and incubating for 1 h at 56 °C. The sample was cooled to room temperature, the DTT solution was replaced with 55 mM iodoacetamide in 100 mM NH<sub>4</sub>HCO<sub>3</sub> (150  $\mu$ l) and then incubated for 45 min in the dark at room temperature, with occasional vortexing. The solution was removed, the gel pieces were washed with 100 mM NH<sub>4</sub>HCO<sub>3</sub> (150  $\mu$ l) and incubated for 10 min at room temperature. The NH<sub>4</sub>HCO<sub>3</sub> solution was replaced with acetonitrile (150  $\mu$ l) to dehydrate the gel pieces and incubated for 10 min

at room temperature. The last two steps were repeated and then the acetonitrile was removed and the sample dried for 10 min using a SpeedVac. The gel pieces were swelled at 4 °C for 45 min in a digestion buffer. The digestion buffer was prepared by dissolving Roche trypsin (modified sequence grade, Roche, Basel, Switzerland) (20 µg) in 50 mM acetic acid (80 µl) and diluting with 50 mM NH<sub>4</sub>HCO<sub>3</sub> to 12.5 ng/µl. The trypsin containing buffer was removed and 50 mM NH<sub>4</sub>HCO<sub>3</sub> (10 µl) was added to keep the gel pieces hydrated during cleavage. The digestion was allowed to proceed overnight at 37 °C, followed by centrifugation for 1 min at 14 000 rpm. The supernatant was stored in a separate Eppendorf tube. The peptides were extracted with 20 mM NH<sub>4</sub>HCO<sub>3</sub> (20 µl) and incubated for 10 min at room temperature. The supernatant was also stored in the Eppendorf tube. Further extractions of the peptides with three changes of 5% formic acid in 50% acetonitrile (25 µl) were performed at room temperature. Finally, the sample was completely dried in a SpeedVac and stored at –20°C until analysed.

### 2.6.3 Electrospray ionisation time-of-flight mass spectrometry

The partially digested peptides were separated on a Waters CapLC instrument (Atlantis, USA) fitted with a dC18 column (150 mm × 100 µm, bead size 3 µm). The peptide samples were loaded in 2% acetonitrile/0.2% formic acid (solvent A), and the column was eluted by using a linear gradient from 3% of solvent B (98% acetonitrile/0.2% formic acid) to 100% of solvent B over 50 min at a flow rate of 1.5 µl/min. Tandem mass spectrometric (MS/MS) data of the eluting peptides were acquired by nano-electrospray quadrupole time-of-flight MS (Waters API Q-TOF Ultima, Massachusetts, USA), scanning from  $m/z$  400 to  $m/z$  1995 at 0.5 s per scan with an interscan delay of 0.1 s. The resulting data were analysed by the Matrix software (<http://www.matrixscience.com>). Fragments were sequence-identified by database searches against publicly available mammalian databases using the Mascot search engine (Perkins *et al.*, 1999) and confirmed using the Basic Local Alignment Search Tool (BLAST; Altschul *et al.*, 1990). Probability-based molecular weight search (MOWSE) (Perkins *et al.*, 1999) scores were estimated by comparing searched results against an estimated random match population and were reported as  $-10 \times \log_{10}(P)$ , with P as the absolute probability.

### 2.6.4 Anion-exchange chromatography

Anion-exchange chromatography on an ÄKTAprime™ (GE Healthcare, Uppsala, Sweden) low pressure liquid chromatograph (LPLC) instrument was used for the purification and separation of amniotic fluid proteins and wool-associated proteins, as described by Armstrong *et al.* (2005). Samples of amniotic fluid and wool were pooled and their protein content estimated according to Bradford (1976), using serum albumin as standard. Each protein sample (1 mg) was desalted and buffer-exchanged on a PD-10 column (Sephadex G-25 medium, GE Healthcare, Uppsala, Sweden) that was previously equilibrated with 25 ml of binding buffer (20 mM Tris-HCl pH 8.8; 5 mM DTT and 1 mM ethylenediaminetetraacetic acid [EDTA]). A 5-ml HiTrap Q strong anion exchange column (GE Healthcare, Uppsala, Sweden) was used for the separation. The column was equilibrated with several column volumes of binding buffer. A linear salt gradient (0–1 M NaCl in binding buffer) was used to elute the bound protein from the column and fractions (1 ml) were collected and pooled as indicated in Fig. 3.96. The separated fractions were loaded on a 10% SDS-PAGE gel as described in § 2.6.1 and identified by comparing their molecular sizes with the previously identified proteins (Fig. 3.92, 3.93 and 3.97). The molecular weight marker used for the wool associated proteins were also used for the proteins separated by anion exchange chromatography (Fermentas PageRuler, Prestained Protein Ladder, #SM0671, Burlington, Canada).

### 2.6.5 Ligand identification

The protein-containing fragments separated by anion exchange chromatography, were combined in glass vials and 500 µl of TBME was added to each fraction (Armstrong *et al.* 2005). The glass vials were sealed and the volatiles extracted from the protein fractions by shaking overnight at 28 °C. The fractions were frozen, the TBME extracts were decanted and then concentrated as described in § 2.3.3.1. The concentrated extracts were conventionally sampled and analysed by GC-MS.

### 2.6.6 Proteolysis

Pronase (Roche, Basel, Switzerland) was dissolved in distilled water (2 ml) and placed in a 15-cm length of high retention, seamless cellulose dialysis tubing (flat width 32 mm x length 200 mm, MW 06205, D0530, Sigma-Aldrich). The enzyme solution was placed in a 3-l glass beaker filled with distilled water and left to dialyse for 48 h. An amniotic fluid sample (20 ml) was allowed to reach room temperature and the enzyme mixture was added to the amniotic fluid in the ratio 10 mg per 20 ml amniotic fluid (1:1 ratio of enzyme to proteins). The sample was then heated at 40 °C for 4 h and enriched with a SEP, as described in § 2.3.3.2.

## 2.7 Statistical Analysis

The peak areas of the target analytes were used to construct data-matrices for the wool collected from day-old lambs in 2007 and 2009, as well as wool collected from week-old lambs in 2007 and for the amniotic fluid collected in 2007. All analytical data were normalised to produce variables with zero means and unit standard deviation (Kowalski and Bender., 1972). Principal component analysis (PCA) biplots, permutation tests and investigations into predictivity were performed using R (Vienna, Austria) as described in Aldrich *et al.* (2004) and Garden-Lubbe *et al.* (2008).

## 2.8 Bioassays

Bioassays were performed during the lambing season of 2009. Single-bearing and twin-bearing ewes were used for bioassays. Within 12 h after parturition single-bearing ewes with their lambs were placed in a 0.2-ha paddock and the twin-bearing ewes, each with her lambs, were isolated in lambing pens (1.2 x 1.6 m) inside a naturally illuminated and ventilated shed. All bioassays were conducted in these pens, or outside in an enclosed concrete-surfaced arena (12 x 8 m). Alien lambs were fitted with disposable diapers (Huggies, New Born, Kimberly-Clark SA, Gardenview, South Africa) and the lambs' bodies were covered from their hind quarters to their shoulder blades with thin, low density polyethylene based plastic (Glad Wrap, Glad/Clorox, Rockdale, Australia) to prevent, or at

---

---

least restrict, odour transfer (Plate 5.1a), after which the lambs were fitted with jackets made from grey or white cotton cloth (fleece cloth) that covered the lambs' bodies from the tail to the back of the neck. The tails of experimental lambs were not tucked into the diapers, but were folded into an extra length of the cloth, which was then fastened with two rubber bands. Openings were made in the jackets for the legs before placing them on the lambs. In some cases jackets with hoods covering the neck and shoulders were used. Openings were cut out for the ears, eyes and mouth of the lambs (Plate 5.1b). The jackets were fastened ventrally using safety pins.

The following experiments were carried out.

1. Synthetic mixtures containing the compounds to be assayed were made up in pentane. In each case the quantitative composition of a synthetic mixture corresponded to the composition of the volatiles present in the headspace of the wool collected from the head of a lamb of the ewe that participated in a particular bioassay. The bioassays were carried out within 24 hours after the wool had been harvested from the lamb. The jackets were sprayed with these synthetic mixtures using a Sigma Spray Kit (No. S 3257, Sigma-Aldrich, St Louis, USA) (Plate 5.1d).
2. Samples of wool (2 g or 8 g) were collected (sheared) from the forehead or the bodies of day-old lambs and Soxhlet-extracted with either DCM, ethyl acetate, pentane or TBME for 1.5 h. These extracts were evenly applied to the fleece jackets and the solvent allowed to evaporate before bioassays were carried out. Small volumes (1 ml) of the extracts were concentrated as described in § 2.3.3.1 for subsequent GC-MS analysis.
3. Wool (8 g) was collected from a day-old lamb and stitched to a jacket using cotton gauze to keep the wool in place. An alien lamb was fitted with this jacket and presented to the mother of the wool-donating lamb.
4. Five lambs were fitted with hoodless fleece jackets and allowed to range with their mothers in a 1-ha paddock for 72 h. The jackets were removed from the lambs and each immediately placed on an alien lamb of approximately the same size and age as the ewe's own lamb. The alien lambs were then presented to the mothers of the lambs that had worn the jackets for the preceding three days. The alien lambs were all accepted practically immediately by the respective ewes. The jackets were then stored separately



at  $-18\text{ }^{\circ}\text{C}$  in glass jars with aluminium foil-backed gastight lids until used in similar bioassays that were carried out at the following times: three, six and nine days, respectively, after they had been removed from the lambs.

- Two lambs were fitted with new jackets and allowed to bond with their mothers, ewes A and B, in the shaded pens (1.2 x 1.6 m) for 72 h. After removal, each jacket was fitted to an alien lamb and the lambs were presented to ewes A and B. Both alien lambs were accepted by the respective ewes. One jacket was extracted with DCM, the extract evenly applied to a new fleece jacket and then the jacket fitted to an alien lamb and presented to ewe A. The other jacket was subjected to SEP sampling of its headspace gas followed by a GC-MS analysis of the trapped material (TIC, Fig. 5.1). The compounds identified in this analysis are listed in Table 3.1.
- Traces of volatile compounds and solvent residues were stripped from lanolin (Wool Fat BP, Medicolab, Johannesburg, South Africa) by steam distillation. Samples of the purified lanolin were dissolved in pentane and then sprayed (Sigma Spray Kit) on two jackets as well as on a jacket tailored from new brown paper. One of the jackets was used in a bioassay and the other one was treated with a synthetic mixture of the compounds identified in the wool headspace. The paper jacket was sprayed with a DCM extract of freshly sheared wool and used in another bioassay.

## 2.9 Synthesis of Reference Compounds

Some of the compounds that were required as reference compounds for the confirmation of the structures of the constituents identified in this study were available from previous studies in LECUS or from the South African distributors of the products of Sigma-Aldrich, Merck, Saarchem, NT Laboratories and B.D.H., and others were synthesised from commercially available starting materials as described below.

The structures of all of the compounds that were synthesised in the present investigation were verified by GC-MS,  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectrometry. Some of the synthesised compounds belong to homologous series. The NMR spectra of compounds

belonging to these homologous series are practically identical and the  $^{13}\text{C}$  NMR data of only one example of each compound class are therefore included in the information below.

### 2.9.1 Preparation of 2,6,10,14-tetramethylhexadecane

A solution of (6*E*,10*E*)-7,11,15-trimethyl-3-methylenehexadeca-1,6,10,14-tetraene (Burger *et al.*, 1978) (0.25 g, 0.92 mmol) in glacial acetic acid (50 ml) was hydrogenated using platinum on activated charcoal (10% Pt/C) as catalyst. The hydrogenation was carried out at approximately atmospheric pressure and the consumption of hydrogen was monitored. The reaction was allowed to run to completion, the reaction mixture was diluted with an equal volume of distilled water and then extracted with  $\text{CHCl}_3$  (3 x 10 ml). The combined extracts were washed with water to neutral pH and dried on anhydrous  $\text{MgSO}_4$ , after which the drying agent was filtered off and the solvent removed on a rotary evaporator to yield 2,6,10,14-tetramethylhexadecane in quantitative yield with a purity of 91% (GC-MS). MS (70 eV):  $m/z$  (%) 282( $\text{M}^+$ , 0.07), 267(0.13), 253(0.17), 197(3), 183(4), 155(3), 141(5), 127(7), 113(8), 99(14), 85(44), 71(84), 57(100), 43(52).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 101 MHz):  $\delta$  = 11.45 (q, C-16), 19.26 (q, C-20)<sup>3</sup>, 19.32 (q, C-19\*)<sup>4</sup>, 19.81 (q, C-18\*), 22.68 (q, C-17\*), 22.77 (q, C-1\*), 24.54 (t, C-8\*), 24.55 (t, C-12\*), 24.86 (t, C-4\*), 28.04 (d, C-2), 29.63 (t, C-15), 32.83 (d, C-6\*), 32.87 (d, C-10\*), 34.49 (d, C-14), 37.01 (t), 37.35 (t), 37.45 (t), 37.46 (t), 37.52 (t), 39.44 (t, C-3).

### 2.9.2 Preparation of branched primary alcohols

Primary isoalcohols and anteisoalcohols were synthesised from the corresponding branched carboxylic acids according to a scaled-down version of the protocol of Tietze and Eicher (1981: 416), as exemplified by the synthesis of 15-methyl-1-hexadecanol. The resulting alcohols were used as starting materials for the preparation of the branched aldehydes described in § 2.9.5.

---

<sup>3</sup> Methyl substituents are numbered in order of their attachment to carbon atoms with ascending numbers in the parent-chain.

*15-Methyl-1-hexadecanol*

15-Methylhexadecanoic acid (8.00 mg; 0.03 mmol) in TBME (200  $\mu$ l) was placed in a 2-ml Reacti-Vial with a small vanned magnetic follower and treated with an excess of a clear standardised solution of LiAlH<sub>4</sub> in dry (Al<sub>2</sub>O<sub>3</sub>, Activity I) diethyl ether. The Reacti-Vial was sealed and the reaction mixture stirred at 60 °C for 3 h. The excess LiAlH<sub>4</sub> was decomposed by the dropwise addition of water (500  $\mu$ l) with stirring. The white precipitate was dissolved by the addition of a few drops of conc. H<sub>2</sub>SO<sub>4</sub> (350  $\mu$ l). The organic phase was separated from the aqueous phase and the aqueous phase extracted with TBME (100  $\mu$ l). The combined extracts were washed to neutral pH with water and dried on anhydrous MgSO<sub>4</sub>. The drying agent was filtered off and the solvent removed on a rotary evaporator to yield 15-methyl-1-hexadecanol (6.00 mg, 79%), with a purity of 95% (GC-MS). MS (70 eV): *m/z* (%) 238(1.3), 210(4), 182(4), 111(25), 97(42), 83(60), 69(78), 57(87), 55(86), 43(100), 41(74).

*8-Methyl-1-nonanol*

MS (70 eV): *m/z* (%) 140(M<sup>+</sup>, 4), 126(14), 112(11), 111(17), 97(14), 85(50), 83(30), 71(51), 70(52), 69(79), 57(74), 56(58), 55(65), 43(100), 41(84).

*11-Methyl-1-tridecanol*

MS (70 eV): *m/z* (%) 196(0.04), 168(1.5), 166(2), 139(4), 125(25), 97(50), 83(72), 71(80), 69(100), 57(85), 55(94), 43(50), 41(62).

*12-Methyl-1-tridecanol*

MS (70 eV): *m/z* (%) 196(0.5), 168(4), 140(6), 125(4), 111(7), 97(33), 83(56), 69(84), 57(84), 56(100), 55(88), 43(93), 41(81).

*13-Methyl-1-tetradecanol*

MS (70 eV): *m/z* (%) 210(0.02), 140(0.2), 125(3), 111(4), 97(18), 83(32), 69(100), 57(33), 56(92), 55(95), 43(72), 41(62).

---

<sup>4</sup> \* Interchangeable assignments.

*14-Methyl-1-pentadecanol*

MS (70 eV):  $m/z$  (%) 224(0.8), 196(4), 168(5), 140(4), 125(6), 111(21), 97(39), 83(59), 69(81), 57(86), 56(86), 55(84), 43(100), 41(78).

*14-Methyl-1-hexadecanol*

MS (70 eV):  $m/z$  (%) 238(0.5), 210(3), 125(13), 111(30), 97(65), 83(79), 70(98), 57(92), 55(100), 43(72), 41(88).

### 2.9.3 Preparation 2-pentadecanol

Following the protocol of Brown and Geoghegan (1967), 1-pentadecene (4.80 g, 0.022 mol) was added to a solution of mercuric acetate (7.01 g, 0.022 mol) in water (22 ml) and purified tetrahydrofuran (22 ml) in a 150-ml round-bottom flask containing a magnetic follower (stir bar). The reaction mixture was stirred for 1.5 h at room temperature to complete the oxymercuration stage. Sodium hydroxide solution (20 ml, 3 M) was then added, followed by a solution of sodium borohydride (20 ml, 0.5 M) in sodium hydroxide (3.0 M). The mercury was allowed to settle and the reaction mixture diluted with purified diethyl ether (20 ml). The organic phase was separated from the aqueous phase and dried on anhydrous  $\text{MgSO}_4$ . The drying agent was filtered off, and the solvent removed on a rotary evaporator to yield 2-pentadecanol (2.88 g, 57%), with a purity of 82% (GC-MS). MS (70 eV):  $m/z$  (%) 210(2), 125(6), 111(8), 97(15), 83(12), 69(12), 57(20), 55(23), 45(100), 43(41). This product was used for the preparation of 2-pentadecanone as described in § 2.9.6.

### 2.9.4 Preparation of 6,10,14-trimethyl-2-pentadecanol

6,10,14-Trimethyl-2-pentadecanone (hexahydrofarnesyl acetone) (50  $\mu\text{l}$ , 0.15 mmol) in TBME (100  $\mu\text{l}$ ) was placed in a 2-ml Reacti-Vial with a magnetic follower and reduced with  $\text{LiAlH}_4$  as described in § 2.9.2 to yield 6,10,14-trimethyl-2-pentadecanol (31.87 mg, 91%), with a purity of 93% (GC-MS). MS (70 eV):  $m/z$  (%) 210(0.3), 196(1), 182(2), 167(1), 140(3), 125(17), 111(24), 97(44), 83(25), 71(63), 69(60), 57(100), 55(70), 45(69), 43(61), 41(38).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 101 MHz):  $\delta$  = 19.70 (q, C-16), 19.77 (q, C-17), 22.64 (q,

C-18\*), 22.74 (q, C-15\*), 23.44 (t, C-8), 23.53 (q, C-1), 24.68 (t, C-4\*), 24.83 (t, C-12\*), 28.00 (d, C-14), 32.79 (d, C-10\*), 32.81 (d, C-6\*), 37.05 (t, C-5\*), 37.15 (t, C-7\*), 37.30 (t, C-9\*), 37.41 (t, C-11\*), 39.39 (t, C-13\*), 39.75 (t, C-3\*), 68.19 (d, C-2).

## 2.9.5 Preparation of aldehydes

Branched and unbranched aldehydes were synthesised by oxidation of the corresponding primary alcohols with pyridinium chlorochromate (PCC) according to Harwood et al. (2003) in yields ranging from 53 to 84%, as exemplified by a microscale preparation of 15-methylhexadecanal from 15-methyl-1-hexadecanol.

### *15-Methylhexadecanal*

15-Methyl-1-hexadecanol (6.00 mg, 0.023 mmol), dissolved in DCM (1 ml) was added dropwise to a magnetically stirred suspension of PCC (7.58 mg, 0.352 mmol) in DCM (200  $\mu$ l) at 0 °C. The reaction mixture was stirred at room temperature for 2 h, diluted with dry diethyl ether (50  $\mu$ l) and the supernatant solution decanted from the black residue. The residue was washed with diethyl ether (3 x 30  $\mu$ l), employing centrifugal separation of the phases, and the combined ether and DCM phases filtered through a short column of silica gel. The solution was dried on anhydrous MgSO<sub>4</sub>, then the drying agent filtered off and the solvent removed on a rotary evaporator to yield 15-methylhexadecanal (5.00 mg, 84%), with a purity of 98% (GC-MS). MS (70 eV): *m/z* (%) 254(M<sup>+</sup>, 0.1), 236(0.3), 210(1), 208(2), 180(2), 154(2), 152(3), 137(4), 123(8), 109(14), 96(23), 95(31), 82(40), 81(43), 69(61), 67(47), 57(100), 55(71), 43(91), 41(62). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 150.88 MHz):  $\delta$  = 22.70 (q, C-16, C-17), 28.01 (d, C-15), 29.10 (t), 29.21 (t), 29.27 (t), 29.39 (t), 29.47 (t), 29.63 (t), 29.67 (t), 29.70 (t), 29.75 (t), 29.98 (t), 33.82 (t, C-3), 39.10 (t, C-14), 43.96 (t, C-2), 203.03 (d, C-1).

### *3-Methylpentanal*

MS (70 eV): *m/z* (%) 100(M<sup>+</sup>, 6), 82(2), 71(19), 58(39), 57(50), 56(100), 44(55), 43(79), 41(86). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75.38 MHz):  $\delta$  = 11.23 (q, C-5), 19.45 (q, C-6), 29.69 (t, C-4), 31.68 (d, C-3), 41.15 (t, C-2), 203.21 (d, C-1).

*8-Methylnonanal*

MS (70 eV):  $m/z$  (%) 138(0.4), 128(4), 114(6), 110(7), 95(34), 82(45), 81(31), 71(31), 69(45), 67(27), 57(93), 55(56), (100), 41(95).

*11-Methyltridecanal*

MS (70 eV):  $m/z$  (%) 212( $M^+$ , 0.1), 194(0.4), 184(0.7), 183(1), 166(2), 165(3), 139(3), 138(2), 137(5), 123(6), 109(26), 96(28), 95(46), 83(42), 82(38), 81(40), 70(59), 69(40), 67(36), 57(95), 55(78), 43(67), 41(100).

*12-Methyltridecanal*

MS (70 eV):  $m/z$  (%) 212( $M^+$ , 0.1), 194(0.4), 184(1), 168(2), 166(2), 153(2), 151(2), 140(3), 138(3), 137(4), 123(7), 110(8), 109(16), 96(20), 95(37), 83(40), 81(47), 71(35), 69(61), 67(45), 57(100), 55(74), 43(92), 41(67).

*13-Methyltetradecanal*

MS (70 eV):  $m/z$  (%) 208(2), 182(3), 180(2), 154(2), 152(2), 137(3), 124(6), 123(6), 109(12), 96(26), 95(28), 82(46), 71(25), 69(43), 68(31), 67(31), 57(79), 55(60), 43(100), 41(83).

*14-Methylpentadecanal*

MS (70 eV):  $m/z$  (%) 222(1), 212(1), 196(1), 194(2), 166(2), 164(2), 152(2), 138(3), 137(4), 124(5), 123(6), 109(14), 96(28), 95(30), 83(31), 82(51), 81(30), 69(43), 57(79), 55(70), 43(100), 41(81).

*14-Methylhexadecanal*

MS (70 eV):  $m/z$  (%) 236(1), 225(1), 208(1), 181(1), 151(2), 137(5), 123(9), 109(21), 96(30), 95(41), 82(45), 81(45), 70(64), 69(49), 67(35), 57(100), 55(79), 43(77), 41(92).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 150.88 MHz):  $\delta$  = 11.44 (q, C-16), 19.26 (q, C-17), 24.73 (t, C-12), 29.10 (t, C-11), 29.27 (t), 29.53 (t, 2 x C), 29.62 (t), 29.67 (t), 29.70 (t), 29.75 (t), 30.06 (t), 31.96 (t, C-3), 34.44 (d, C-14), 36.68 (t, C-13), 43.95 (t, C-2), 203.00 (d, C-1).

*Tetradecanal*

MS (70 eV):  $m/z$  (%) 212( $M^+$ , 0.13), 194(1), 184(1), 168(5), 166(3), 138(7), 124(9), 110(38), 96(32), 82(38), 69(27), 68(28), 67(28), 57(82), 55(77), 43(100), 41(95).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 101 MHz):  $\delta$  = 14.11 (q, C-14), 22.11 (t, C-13), 22.71 (t, C-12), 29.09 (t), 29.19 (t), 29.27 (t), 29.38 (t), 29.45 (t), 29.61 (t), 29.67 (t), 29.69 (t), 31.94 (t, C-3), 43.92 (t, C-2), 203.00 (d, C-1).

*Pentadecanal*

MS (70 eV):  $m/z$  (%) 226( $M^+$ , 0.2), 208(2), 182(4), 180(3), 152(5), 138(6), 124(8), 110(14), 96(40), 82(44), 69(22), 68(23), 67(22), 57(65), 55(61), 43(93), 41(100).

*Hexadecanal*

MS (70 eV):  $m/z$  (%) 240( $M^+$ , 0.2), 222(2), 194(3), 166(3), 152(3), 138(6), 124(10), 110(16), 109(15), 96(45), 82(53), 69(25), 68(26), 67(25), 57(61), 55(58), 43(100), 41(94).

*Heptadecanal*

MS (70 eV):  $m/z$  (%) 254( $M^+$ , 0.2), 236(2), 210(3), 208(3), 180(2), 152(3), 138(6), 137(5), 124(10), 123(8), 110(15), 109(14), 96(43), 82(47), 69(23), 68(23), 67(22), 57(60), 55(57), 43(100), 41(85).

## 2.9.6 Preparation of methyl ketones

Methylketones were prepared by PCC oxidation according to Harwood et al. (2003) from the corresponding secondary alcohols, as described in section §. 2.9.5.

*2-Tetradecanone*

MS (70 eV):  $m/z$  (%) 212( $M^+$ , 4), 197(2), 154(4), 153(3), 127(2), 111(2), 110(2), 96(6), 85(11), 71(42), 59(48), 58(100), 55(18), 43(96), 41(33).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75.38 MHz):  $\delta$  = 14.10 (q, C-14), 22.68 (t, C-13), 23.88 (t, C-4), 29.18 (t), 29.35 (t), 29.40 (t), 29.47 (t), 29.53 (t), 29.61 (t), 29.65 (q, C-1), 29.81 (t), 31.92 (t, C-12), 43.81 (t, C-3), 209.30 (d, C-2).

*2-Pentadecanone*

MS (70 eV):  $m/z$  (%) 226( $M^+$ , 7), 211(3), 168(6), 138(3), 127(5), 110(5), 96(11), 85(17), 71(39), 59(68), 58(92), 43(100), 41(55).

### 2.9.7 Preparation of 6,10-dimethyl-2-undecanone

6,10-Dimethyl-5,9-undecadien-2-one (geranylacetone) (0.25 g, 1.29 mmol) was hydrogenated in glacial acetic acid (50 ml) using platinum on activated charcoal (10% Pt/C) as catalyst at atmospheric pressure. The consumption of hydrogen was monitored and was terminated after the consumption of a volume of hydrogen equivalent to the reduction of two double bonds. The reaction mixture was then diluted with an equal volume of distilled water and extracted with  $\text{CHCl}_3$  (3 x 10 ml). The combined extracts were washed with water to neutral pH and dried on anhydrous  $\text{MgSO}_4$ , after which the drying agent was filtered off and the solvent removed on a rotary evaporator to yield 6,10-dimethyl-2-undecanone (0.24 g, 95%), with a purity of 88% (GC-MS). MS (70 eV):  $m/z$  (%) 198( $M^+$ , 0.4), 180(4), 140(3), 123(3), 109(11), 95(11), 85(16), 71(28), 58(77), 43(100), 41(23).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100.58 MHz):  $\delta$  = 19.55 (q, C-12), 21.44 (t, C-4), 22.69 (q, 2 x C), 24.73 (t, C-8), 27.96 (d, C-10), 29.83 (q, C-1), 32.65 (d, C-6), 36.52 (t, C-7), 37.10 (t, C-5), 39.32 (t, C-9), 44.14 (t, C-3), 209.35 (d, C-2).





a. Collection of amniotic fluid from a newborn lamb using a stainless steel spoon.



b. Collection of amniotic fluid in an Eppendorf tube for protein analyses.



c. Collection of wool samples.



d. SEP50 installed in a 50-ml glass bottle containing amniotic fluid.



e. SEP50 installed in a 50-ml glass bottle containing lamb's wool.



f. SEP50 installed in a 8-ml glass bottle containing lamb's wool.

## Plate 2.1

---

---

## References

Aldrich, C., Gardner, S. and Le Roux, N. J., "Monitoring of metallurgical process plants using biplots", *J. Am. Inst. Chem. Eng.* **50**: 2167–2186 (2004).

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J., "Basic local alignment search tool", *J. Mol. Biol.* **215**: 403–410 (1990).

Armstrong, S. D., Robertson, D. H. L., Cheetham, S. A., Hurst, J. L. and Beynon, R. J., "Structural and functional differences in isoforms of mouse major urinary proteins: a male-specific protein that preferentially binds a male pheromone", *J. Biochem.* **391**: 343–350 (2005).

Bradford, M. M., "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding", *Anal. Biochem.* **72**: 248–254 (1976).

Brown, H. C. and Geoghegan, P., "The oxymercuration-demercuration of representative olefins. A convenient, mild procedure for the Markovnikov hydration of the carbon-carbon double bond", *J. Am. Chem. Soc.* **89**: 1522–1524 (1967).

Burger, B. V., Le Roux, M., Spies, H. S. C., Truter, V. and Bigalke, R. C., "Mammalian pheromone studies, III. (*E,E*)-7,11,15-Trimethyl-3-methylenehexadeca-1,6,10,14-tetraene, a new diterpene analogue of  $\beta$ -farnesene from the dorsal gland of the springbok, *Antidorcas marsupialis*", *Tetrahedron Lett.* **52**: 5221–5224 (1978).

Burger, B.V., Marx, B., Le Roux, M. and Burger, W. J. G., "Simplified analysis of organic compounds in headspace and aqueous samples by high-capacity sample enrichment probe", *J. Chrom. A* **1121**: 259–267 (2006).

---

---

Burger, B.V., Viviers, M. Z., Bekker, J. P. I., Le Roux, M., Fish, N., Fourie, W.B. and Weibchen, G., "Chemical characterization of territorial marking fluid of the male Bengal tiger, *Panthera tigris*", *J. Chem. Ecol.* **34**: 659–671 (2008).

Gardner-Lubbe, S., Le Roux, N. J. and Gower, J. C., "Measures of fit in principal component and canonical variate analyses", *J. Appl. Statist.* **35**: 947–965 (2008).

Grob, K., "Organic substances in potable water and in its precursor. Part 1. Methods for their determination by gas-liquid chromatography", *J. Chromatogr. B* **84**: 255–273 (1973).

Harwood, L. M., Moody, C. J. and Percy, J. M., "Experimental Organic Chemistry. Standard and Microscale", Second Edition, Blackwell Science, Germany (2003). Exp. 31, pp 507–510.

Mascot search engine, Matrix Science Ltd (2007) <http://www.matrixscience.com/cgi/>.

Kovats, E., "Gas-chromatographische charakterisierung organischer verbindungen. Teil 1: retentionsindices aliphatischer halogenide, alkohole, aldehyde und ketone", *Helv. Chim. Acta* **41**: 1915–1932 (1958).

Kowalski, B. R. and Bender, C. F., "Pattern recognition. A powerful approach to interpreting chemical data", *J. Am. Chem. Soc.* **94**: 5632–5639 (1972).

Laemmli, U. K., "Cleavage of structural proteins during the assembly of the head of bacteriophage T4", *Nature* **227**: 680–685 (1970).

Maas, B., Dietrich, A. and Mosandl, A., "Collection of enantiomer separation factors obtained by capillary gas chromatography on chiral stationary phases", *J. High Resolut. Chrom.* **17**: 109–115 (1994).

---

---

Perkins, D. N., Pappin, D. J. C., Creasy, D. M. and Cottrell, J. S., "Probability-based protein identification by searching sequence databases using mass spectrometry data", *Electrophoresis* **20**: 3551–3567 (1999).

R Development Core Team (2009). *R: A Language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria. URL <http://www.R-project.org>.

Reiter, B., Burger, B. V. and Dry, J., "Mammalian exocrine secretions XVIII. hemical characterization of interdigital secretion of red hartebeest, *Alcelaphus buselaphus caama*", *J. Chem. Ecol.* **29**: 2235-2252 (2003).

Sambrook, J. and Russell, D. W., "Molecular Cloning: A Laboratory Manual", Third Edition, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York (2001).

Shevchenko, A., Wilm, M., Vorm, O. and Mann, M., "Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels", *Anal. Chem.* **68**: 850–858 (1996).

Tietze, L.-F. and Eicher, T., "Reaktionen und Synthesen im organisch-chemischen Praktikum", Georg Thieme, Stuttgart, Germany (1981).

Vincenti, M., Guglielmetti, G., Cassani, G. and Tonini, C., "Determination of double-bond position in diunsaturated compounds by mass spectrometry of dimethyl disulfide derivatives", *Anal. Chem.* **59**: 694–699 (1987).

## CHAPTER 3

# CHEMICAL CHARACTERISATION OF THE COMPOUNDS PRESENT IN THE WOOL AND THE AMNIOTIC FLUID OF DÖHNE MERINO SHEEP, *OVIS ARIES*

### 3.1 Chemical Characterisation of the Volatile Organic Fractions of the Wool and the Amniotic Fluid

#### 3.1.1 Introduction

The characterisation of the VOCs present in the wool of the lambs and the testing of mixtures of their synthetic analogues in add-on fostering experiments were the main objectives of this investigation. Because the importance of amniotic fluid in maternal behaviour in sheep is well documented, the possibility that amniotic fluid could also be a source of the individual olfactory cues responsible for ewe–lamb bonding was also considered. The identification of the VOCs present in amniotic fluid of ewes will therefore also be briefly discussed and all the identified constituents are shown in Table 3.1.

#### 3.1.2 Analytical methods

##### 3.1.2.1 Gas chromatography-mass spectrometry

A detailed description of the instrumentation and procedures that were used to analyse the VOCs of wool and amniotic fluid samples collected during the lambing season of 2007 is given in Chapter 2. Here the application of those analytical methods to the identification of the VOCs of the collected wool and amniotic fluid samples will be discussed. GC-MS analyses of the wool headspace samples and the amniotic fluid extracts on PS089-OH and AT-1000 coated capillary columns, using the standard GC parameters resulted in satisfactory separation of the VOCs present in both the wool and the amniotic fluid. Typical examples of total ion chromatograms (TICs) obtained in GC-MS analyses of wool and amniotic fluid samples are depicted in Figs. 3.1 and 3.2, respectively.

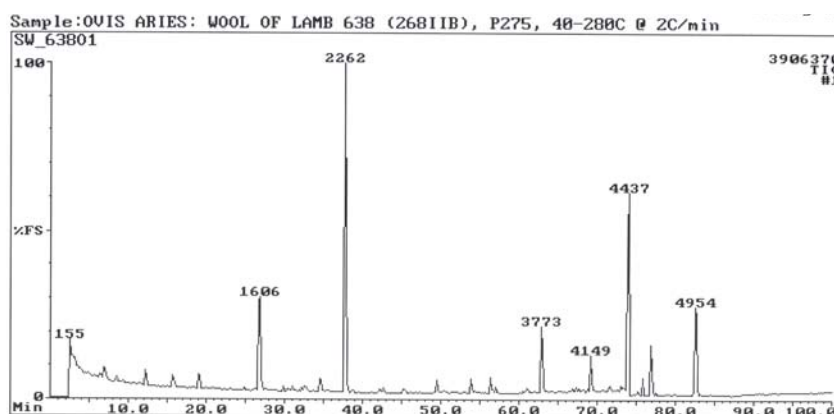


Fig. 3.1. TIC obtained by GC-EIMS analysis of the VOCs extracted with SEP50 from the wool collected from lamb US-2007-0638.<sup>5</sup>

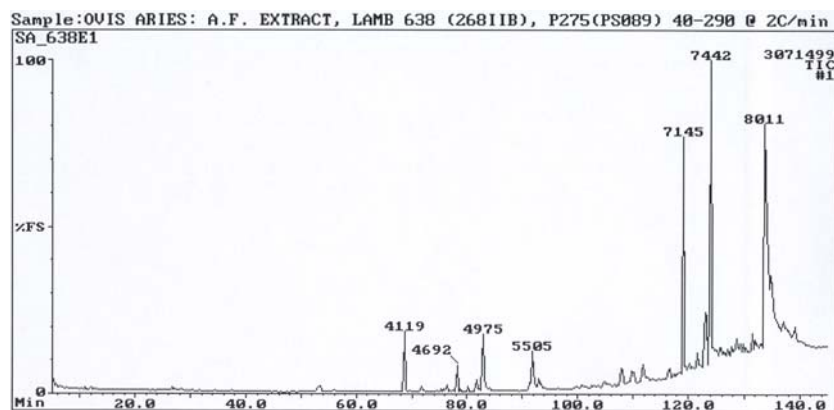


Fig. 3.2. TIC obtained by GC-MS analysis of the VOCs extracted with TBME of the amniotic fluid collected from the body of lamb US-2007-0638.

### 3.1.2.2 High-resolution gas chromatography-mass spectrometry

Gas chromatographic separation of the constituents of complex mixtures in conjunction with high-resolution mass spectrometry (GC-HRMS) can simplify the interpretation of mass spectra by providing the elemental composition of the molecular ion and of other ions that could provide important diagnostic information. HRMS data can also be used to provide information on the position of double bonds in long-chain compounds that are often present in mammalian exocrine secretions. If an unsaturated long-chain compound

<sup>5</sup> All the TICs depicted in this chapter were obtained in GC-EIMS analyses using Column A and the analytical conditions given in Chapter 2 (§ 2.3).

has a mass spectrum in which the hetero-atoms of the functional group are retained in most of the abundant ions, then a mass spectrum containing only these ions can be constructed to provide information on the location of the double bond in the compound. If the normal succession of abundant ions is interrupted, it can be construed as evidence that a double bond is present at the corresponding position in the molecule. In certain cases the feature can be used effectively to determine the position of branching in a molecule (Burger, 2005b). The gas chromatograph-time-of-flight mass spectrometer (GC-TOF-HRMS) that was available during the later stages of the present investigation proved to be more sensitive than the quadrupole instruments that were initially used. The information obtained with the former instrument was used mainly to confirm the qualitative information that had already been obtained with the quadrupole instruments. It also provided valuable information on the presence and composition of molecular ions that could previously not be detected in the mass spectra of some of the constituents present in trace concentrations in the samples that were analysed.

The high data acquisition rate of the instrument also allowed deconvolution of overlapping peaks in the TIC. This made high quality mass spectral information available and it was thus possible to identify compounds that would otherwise have remained unidentified. In high-resolution analyses with this instrument mass differences of less than or equal to 2 mDa between the observed mass and the mass calculated for a specific ion were considered acceptable.

### 3.1.2.3 Enantioselective gas chromatography-mass spectrometry

Because LECUS has a wide range of enantioselective gas chromatographic columns available, enantioselective gas chromatography was the method of choice for the determination of the absolute configuration of the chiral constituents of the wool and the amniotic fluid samples. The best results were obtained with glass columns coated with OV-1701 containing 10% of either heptakis(2,3-di-O-methyl-6-O-tert-butyldimethylsilyl)- $\beta$ -cyclodextrin (column C) or heptakis(2,3-di-O-acetyl-6-O-tert-butyldimethylsilyl)- $\beta$ -cyclodextrin (column D) as chiral selectors. OV-1701 is a moderately polar stationary phase

---

---

from which even the lower carboxylic acids elute as reasonably sharp peaks and the higher acids as peaks with the peak widths of apolar compounds.

#### 3.1.2.4 Determination of double bond positions

Off-line DMDS derivatisation of unsaturated compounds and GC-MS analysis of the derivatives (Vincenti *et al.*, 1987) was used to determine the positions of double bonds in the unsaturated constituents of the wool and amniotic fluid samples. This method, successfully used since 1981, involves the iodine catalysed reaction of double bonds with DMDS and GC-MS analysis of the resulting adducts. Although this reaction takes at least 24 h to run to completion, quantitative derivatisation of compounds with a wide variety of functional groups can be attained. The addition of DMDS to the double bond is stereospecific and the adducts of the *Z*- and *E*- isomers of a compound can be separated by GC (Tien, 1993).

The advantage of off-line derivatisation techniques is that multistep procedures including removal of solvents, reagents and by-products are possible, but the disadvantage of this technique is that, under certain circumstances, detection of derivatised compounds may be difficult. Problems may be encountered if the target compound is a relatively minor constituent of a complex mixture, and its derivatised product co-elutes with another constituent (Smit, 1997: 54–56). This was the case for most of the unsaturated compounds present in the wool and the amniotic fluid samples because they were present in very small quantities. Another problem inherent to this technique is that the derivatised compounds have significantly higher masses than the underivatised compounds. The compounds might therefore not elute from the column, or they could decompose on the column. DMDS derivatisation can nevertheless provide invaluable structural information.

For some reason the majority of unsaturated compounds present in the wool and the amniotic fluid samples were not quantitatively derivatised and appeared unchanged in the TIC of the mixture of reaction products. Furthermore, some minor unsaturated constituents were not detected in the reaction product mixture, either as the unchanged compounds or as their derivatives. Even when using a larger sample of the extract, a larger excess of the



reagents and longer reaction times, the derivatives of the major unsaturated compounds were still formed in very low yields.

### 3.1.3 Chemical characterisation of the volatile organic fraction of the wool samples

#### 3.1.3.1 Introduction

There are several sources from which the VOCs constituting each lamb's unique olfactory cue could conceivably be released. The odour signature of a lamb could originate from the ewe, *i.e.* it can be a maternal label. Maternal labels can originate from the amniotic fluid, milk, saliva, or some other secretion or excretion that could be transferred from ewe to lamb. Three possible sources of maternal labels in sheep, namely colostrum, the amniotic fluid and the inguinal gland secretion, have been investigated by Rietdorf (2002). Environmental factors might also come into play, although this is unlikely, because the great number of individuals in a flock creates practical problems for the creation of unique odour signatures if the flock of sheep inhabit the same area. The other alternative is that each lamb is born with its own individual odour profile, and that the amniotic fluid is merely a role player in the greater recognition process: initiating interest of the ewe in her lamb and inducing licking and grooming behaviour so that the ewe can learn to recognise the lamb's olfactory profile. The interest that ewes display in the lambs' hindquarters is well documented in the literature (Alexander *et al.*, 1983; Houpt, 2005: 195; Lynch *et al.*, 1992: 148), and the interest that ewes show for the lambs' head and neck areas has also been noted (Price *et al.*, 1984). However, during the lambing season of 2007 it became abundantly clear that in general ewes showed no preference for a specific area on lambs' bodies when sniffing their lambs during the initial identification process. A ewe would inspect that part of a lamb closest to her for initial recognition. The lamb is finally accepted at the udder with its head turned away from the head of the ewe and at that point the ewe starts showing more interest in the lamb's hindquarters. In Plates 3.1a to 3.1f and 3.2a to 3.2e the different areas that the ewes investigated during the initial recognition process, are illustrated. These observations confirmed the results of the experiments done by Alexander and Stevens (1981), Brennan and Kendrick (2006) and Lynch *et al.* (1992: 148), who found that the discrimination of ewes

---

---

between their own lambs and alien lambs is mediated primarily by olfactory cues from the lamb's wool and skin, not a specific area on the lamb's body, and also not from the amniotic fluid. Furthermore, Kendrick *et al.* (1992) found that lamb odours had no influence on the activity of the olfactory bulb neurons of ewes during the period before birth, but when tested in the period after birth the lamb odours were the most potent olfactory stimulus for the ewes. More importantly, for all ewes tested, the smell of the lamb's wool was almost as effective a stimulus as the smell of the whole lamb.

The detection of the unique olfactory signatures necessary for the ewes to recognise their lambs which are possibly present in the wool, can either be facilitated by the MOS or the AOS; the MOS detects volatile odours in the air and the AOS typically detects non-volatile cues sampled through direct contact (Dulac and Torello, 2003). The MOS is ideally suited for the reorganisation of profiles of airborne volatiles that constitute an individual odour signature, as described in Chapter 1 (§ 1.2.3.1) (Brennan and Kendrick, 2006). However, the roles played by the MOS and the AOS in individual recognition are currently still controversial. Given that Lévy *et al.* (2004) have shown that ewes can recognise their lambs through wire cages, without any direct contact with their lambs, and with the only cues available for olfactory recognition being volatile molecules, it was decided to focus the present research on the identification of volatile semiochemicals. Furthermore, it has been established that the cues responsible for the individual olfactory signature of lambs and goat kids are not effective at distances greater than 25 cm (Alexander, 1978; Alexander and Shillito, 1977; Poindron *et al.*, 2003). This suggests that the olfactory cues are either not very volatile or they are present in very low concentrations (Poindron *et al.*, 2006).

After considering all of the above-mentioned literature and, most importantly, the observation made during the lambing season of 2007 at Mariendahl, the present investigation was focused on the wool of lambs as the possible source of each lamb's individual odour profile.

### 3.1.3.2 Sample collection and preparation

A ewe flock of Döhne Merinos was made available for the collection of samples and bioassays at Mariendahl, an experimental farm of the Stellenbosch University, located 14 km outside Stellenbosch. Single- and twin-bearing ewes were housed in separate paddocks. A total of 16 of the 165 twin-bearing ewes were used as a sample group (9.69%). Wool samples were collected from these ewes and their lambs. The following were recorded: stud number of the ewe and her twin lambs, the gender of the lambs and the order in which the lambs were born.

Cranial wool samples were collected from 16 twins (Plate 2.1c) the morning after birth and again seven days later from five of the same 16 twins. This was done to track the development of the odour profiles of the lambs. As mentioned in Chapter 2, all the animals were maintained in a computerised flock-recording scheme, using the program Shepherd (Olivier, 2003). This made it possible to compare the constituents present in each lamb's wool with those of the lamb's closest kin as well as with those of non-related lambs. It also made similar comparisons possible regarding the odour profiles of the offspring of ewes in different familial relationships. It was thus anticipated that genetic influences on lamb odours could possibly be studied by comparing odour profiles of closely related lambs with those of non-related lambs.

The same ewe flock was used for the collection of wool two years later. Because it was agreed that the present investigation should not interfere with the long-term breeding programme at Mariendahl, only five ewes that lambed in 2007 were, unfortunately, available for sample collection in 2009, and none of the rams used in 2007 were again used in 2009. Although the sample group was considerably reduced, the information gained from investigating the odour profile of lambs from the same ewe two year later was expected to provide valuable insight into whether the odour is linked to genetic trends. In other words, do the odour profiles of the lambs born during 2007 and in 2009 from the same ewes, whether twins or not, show closer resemblance than the odour profiles of lambs from any other ewes in the flock.

### 3.1.3.3 Sampling techniques

The analyses carried out with the wool samples collected in 2007 is summarised in Fig. 2.2. Headspace analyses were carried out to identify the VOCs present in the wool samples. Several enrichment techniques were investigated to determine the best headspace analytical method and parameters for this purpose. Each of these techniques will be briefly discussed.

#### a) Solid-phase micro-extraction

Although excellent results have been obtained in many applications of the SEP sample enrichment technique in LECUS, SPME was now investigated as an alternative enrichment technique because of the simplicity of the technique and the shorter time needed for equilibrium sampling. However, the much higher capacity of the SEP technique produced results that were clearly superior (Figs. 3.3 and 3.4).

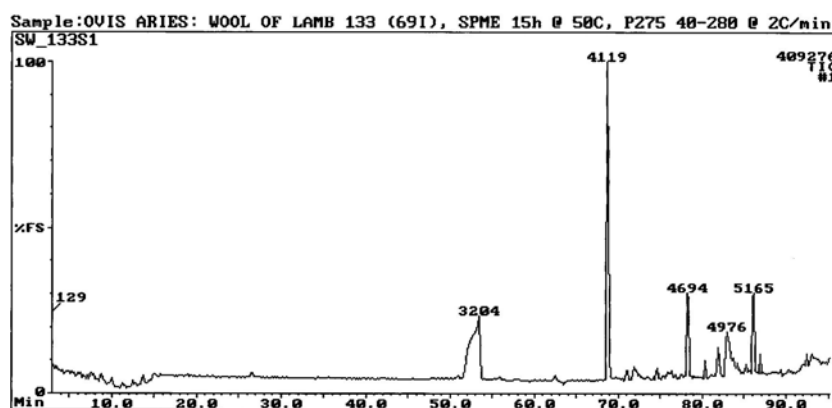


Fig. 3.3. TIC obtained by GC-MS analysis of the VOCs collected by SPME from the wool of lamb US-2007-0133.

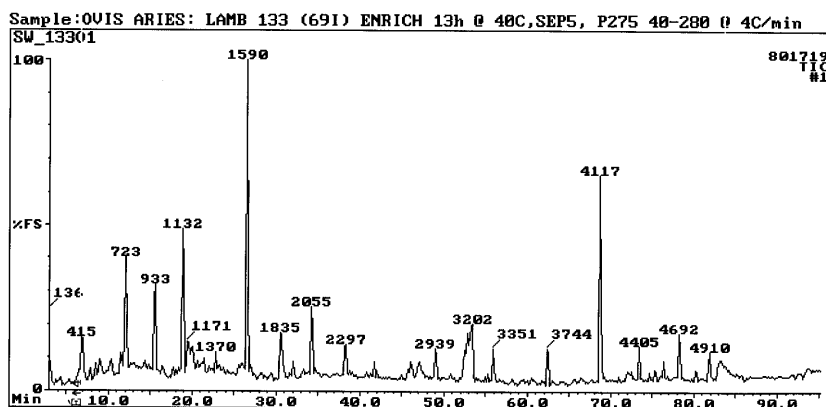


Fig. 3.4. TIC obtained by GC-MS analysis of the VOCs collected by SEP50 from the wool of lamb US-2007-0133.

b) Headspace enrichment with the sample enrichment probe technique

When the SEP technique was introduced in 2006 (Burger *et al.*) it was mentioned that the reproducibility [relative standard deviation (RSD)] that can be achieved in quantitative analyses with this technique was not satisfactory. However, SEPs of the second generation that were used in the present study give RSDs better than 5% (Burger, unpublished results).

The practical application of the SEP technique in the sampling of the wool volatiles is described in § 2.3.3.2. In the first exploratory experiment SEP enrichment carried out over 2.75 h at 40 °C and GC-MS analysis of the volatiles present in the wool gave the TIC shown in Fig. 3.5. Under these conditions the sensitivity of the SEP technique was disappointingly low and only a few peaks were visible in the TIC. After substituting the 50-ml glass sampling bottles that were initially used with 8-ml glass vials, which necessitated the reduction of the wool sample from 400 mg to 70 mg, slightly better results were obtained. Increasing the duration of the enrichment to 6 h did not result in an appreciable improvement of the sensitivity of this static enrichment method.

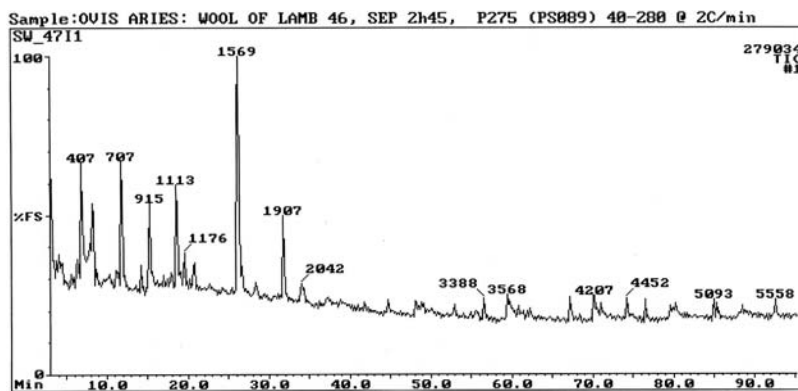


Fig. 3.5. TIC obtained by GC-MS analysis of the VOCs collected with a SEP50 over 2.75 h at 40 °C from *ca.* 200 mg of the wool of lamb US-2007-0046.

In order to increase the efficiency of the enrichment process the use of dynamic closed-loop stripping in conjunction with SEP trapping of the volatiles was explored, as described in § 2.3.3.2. Dynamic enrichment gave an approximately 70% increase in the quantities of the volatiles trapped with static SEP enrichment. However, a serious problem became apparent when it was discovered that the contamination of the air-circulating pump with material from the wool could not be removed by pumping clean air through the system. Even rinsing the pump with DCM, methanol, acetone and water, followed by overnight pumping of air, was not totally effective in cleaning the pump. Because such a cleaning process would also have been too time-consuming for routine sample enrichment, this technique was abandoned.

Although it is known that the only benefit of microwave radiation would be the heating of the sample (Herrero *et al.*, 2008), this method was investigated as an alternative possibility of increasing the sensitivity of the SEP technique. It was thought that by heating moisture adsorbed on the wool the organic compounds on the wool fibres could be volatilised in a process that could perhaps be described as micro steam distillation. PDMS rubber is not susceptible to microwave irradiation. In a microwave oven the rubber remains cold, at least for the short while before it is heated by any hot water vapour that happens to be present. It was hoped that the temperature gradient between the hot wool fibre surfaces and the cooler rubber would promote sorption of the volatiles into the rubber. Experiments were conducted in a domestic microwave oven for various periods of time and at different power settings

(Watt), using constant and pulsed heating cycles. Continuous heating proved to be more efficient than pulsed heating. Increasing the enrichment time as well as decreasing the power in order to avoid excessive heating of the SEP's rubber sleeve proved to be less efficient. Using a series of crystalline organic compounds with different melting points as a method to determine the temperature in the microwave oven, it was found that the air temperature reached about 145 °C when the appliance was operated at 600 W for 5 min. Similar enrichments carried out in a conventional electrically heated oven produced results that were clearly superior to those obtained using the microwave oven. Attempts at using microwave heating in SEP analyses were therefore discontinued.

However, these experiments did confirm the known beneficial effect of heating the sample in sorptive sample enrichment techniques. Furthermore, an enrichment temperature of 145 °C was considered to be unnecessarily high. In experiments carried out at sample temperatures up to 140 °C for periods from 5 to 60 min the best results were obtained by heating the sample at 70 °C in a small vial for 60 min (Figs. 3.6 to 3.9).

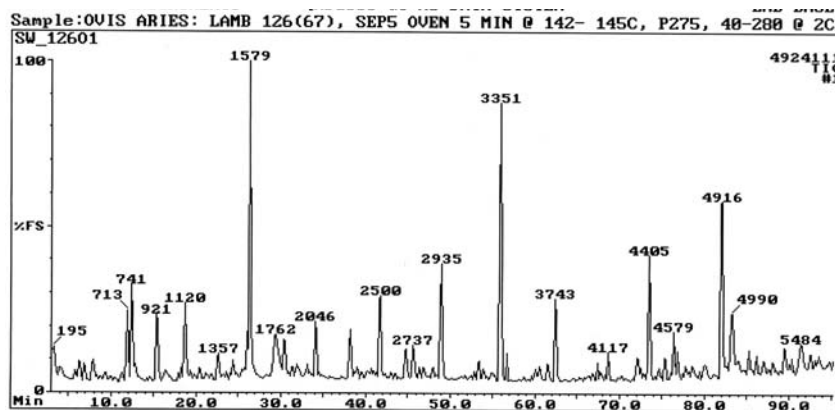


Fig. 3.6. TIC obtained by GC-MS analysis of the VOCs collected with a SEP50 for 5 min at 145 °C from 70 mg of the wool of lamb US-2007-0126.

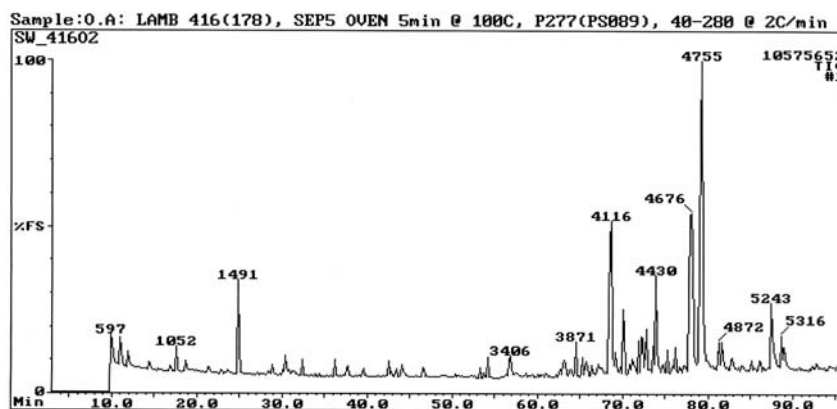


Fig. 3.7. TIC obtained by GC-MS analysis of the VOCs collected with a SEP50 for 5 min at 100 °C from 70 mg of the wool of lamb US-2007-0416.

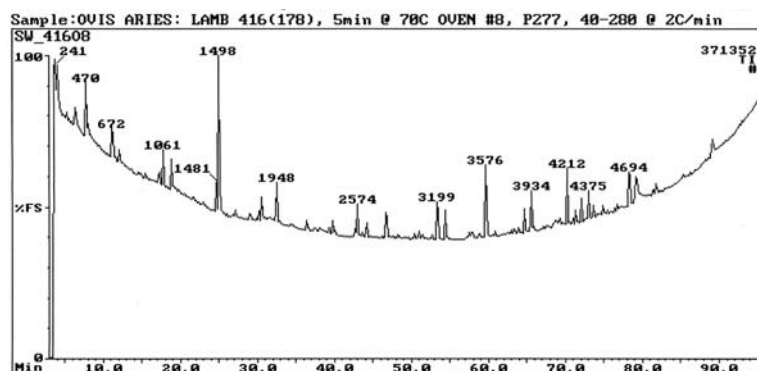


Fig. 3.8. TIC obtained by GC-MS analysis of the VOCs collected with a SEP50 for 5 min at 70 °C from 70 mg of the wool of lamb US-2007-0416.

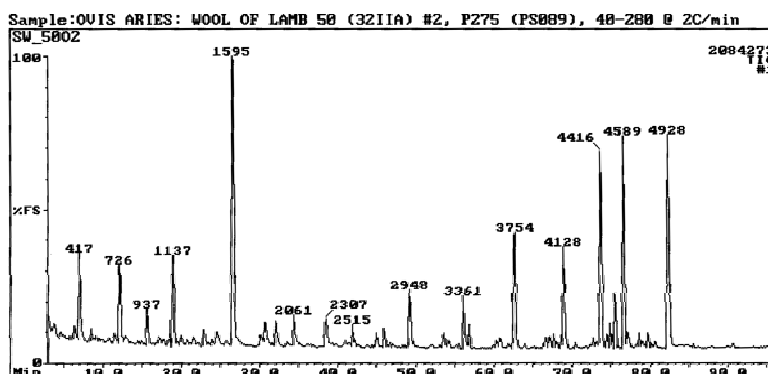


Fig. 3.9. TIC obtained by GC-MS analysis of the VOCs collected with a SEP50 for 60 min at 70 °C from 70 mg of the wool of a lamb US-2007-0050.



## c) Conventional sample extraction

Conventional sample extraction, concentration of the extracts and GC and GC-MS analyses were also employed in the identification of the volatile constituents present in the wool samples collected from the experimental animals. This was done in order to ensure that compounds that are for some reason not sorbed into the PDMS rubber as efficiently as others would nevertheless be detected and quantified. Unfortunately, in a situation like this, an ideal solvent that could be expected to extract all the (unknown) analytes equally well, does not exist. In view of the successful extraction of volatiles from the amniotic fluid, it was decided to use TBME for the extraction of the wool of the lambs as described in § 2.3.3.1. The TIC of the extract a typical wool sample using column A is shown in Fig. 3.10.

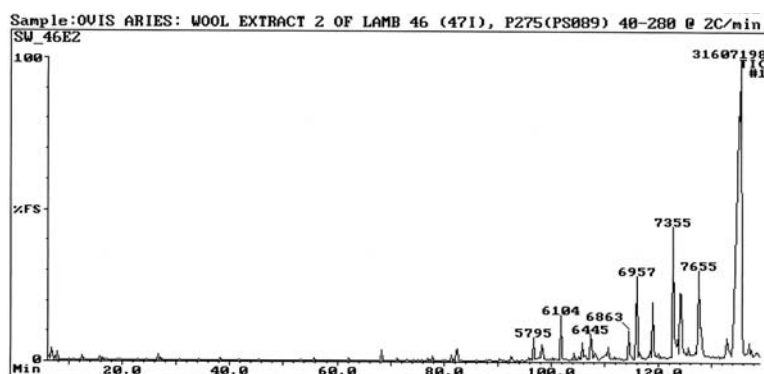


Fig. 3.10. TIC obtained by GC-MS analysis of the TBME extract of the wool of lamb US-2007-0046.

Although the SEP technique has its drawbacks, notably regarding its discrimination against highly polar compounds, it was expected to provide a more accurate picture of the composition of the volatiles released into the atmosphere from a wool sample; also because it is almost impossible to concentrate extracts without losing a considerable proportion of the most volatile constituents of an extract. Another advantage of the SEP technique is that the column is subjected to less thermal stress because it is not necessary to programme the column to very high temperatures to elute high boiling constituents present in an extract. These compounds that probably do not play an important role in the chemical communication of the species under investigation, besides possibility being involved in the controlled release of the pheromone. On the other hand, analysing an extract could furnish

---

---

information on how a synthetic sample should be formulated to produce an effluvium plume corresponding to the results obtained with the SEP technique. Results obtained for SEP analyses of wool samples are presented and discussed in section § 3.1.5.

### 3.1.4 Chemical characterisation of the volatile organic fraction of the amniotic fluid

#### 3.1.4.1 Introduction

Ewes display a strong attraction to amniotic fluids a few hours before and after parturition and it is this attraction that results in licking and grooming of the lamb (Plates 3.1a and 3.1b), low pitched bleating and the acceptance of the lamb at the udder. These behavioural patterns are conducive to the formation of the exclusive bond between the ewe and lamb, and depend on the ewe's ability to memorise the individual olfactory cues from her offspring (Houpt, 2005: 192; Lynch *et al.*, 1992: 135). Even though Lévy *et al.* (2004) suggest that amniotic fluid contains cues for general attractiveness rather than for individual recognition, and that amniotic fluid reinforces maternal behaviour, it was considered important to investigate the possibility that residual amniotic fluid left on the lamb's body after it has been groomed by its mother could be the origin of the lamb's individual olfactory cue in the bonding process.

#### 3.1.4.2 Sample collection and preparation

The same sample group of animals selected for the collection of the wool samples (§ 3.1.3.2) was also used for the collection of ewes' amniotic fluid samples. This was done so that the compounds identified in the wool of each lamb could also be compared with the compounds identified in the amniotic fluid of its mother. Amniotic fluid was scraped off the body of a newborn lamb with a stainless steel spoon (Plate 2.1a) directly after the ewe had given birth and then stored in glass bottles with Teflon-faced screw caps. The utilisation of samples for GC-MS analysis from the collected amniotic fluid samples is summarised in Fig. 2.1.

### 3.1.4.3 Sampling techniques

The first approach used to investigate the role of the amniotic fluid volatiles as chemical messengers for kin recognition, was to identify the volatiles present in the headspace of the amniotic fluid. Initially, samples of amniotic fluid were stirred and heated at 40 °C during headspace sampling to facilitate fast transport of the volatiles from the liquid to the gas phase for sorptive enrichment in the PDMS sleeve of the SEP. However, when some samples were removed from the oven after 4 h the initially liquid amniotic fluid had turned into thick gels, which could not be stirred. The formation of the gel could probably be ascribed to denaturing of proteins present in the fluid. Only a few volatiles enriched from the upper layer of the gel were present in the resulting TIC (Fig. 3.11). Although not all of the samples formed gels, this phenomenon resulted in unacceptably low levels of enrichment in the affected samples.

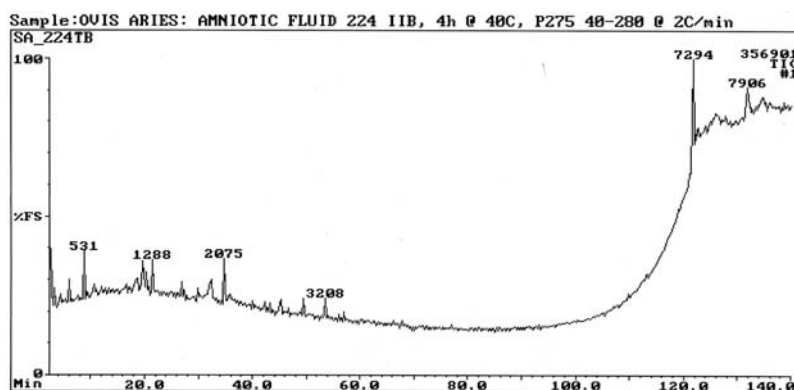


Fig. 3.11. TIC obtained by GC-MS analysis of the VOCs collected with a SEP50 at 40 °C of the amniotic fluid collected from the lamb US-2007-526.

To decrease the viscosity of the amniotic fluid in order to ensure efficient stirring and sample enrichment, an enzyme was added to the amniotic fluid samples. Pronase, a non-specific mixture of proteinases known to break down both denatured and native proteins into individual amino acids (Narahashi, 1970), was used for this purpose. Sample enrichment was allowed to proceed for 4 h at 40 °C. On removal of the SEP after enrichment, the sample had a strong sulphurous smell. The TIC revealed the presence of numerous compounds, including a range of sulphur-containing compounds and amines that were not detected in amniotic fluid samples that were not treated with pronase. This was probably caused by the

metabolic activity of the microbiota introduced into the amniotic fluid at birth on the higher concentration of free amino acids made accessible by the pronase. The same phenomenon has been observed when sausages were treated with pronase (Díaz *et al.*, 1993; Hagen *et al.*, 1996; Herranz *et al.*, 2006).

Following these unsuccessful attempts at trapping the amniotic fluid headspace volatiles for GC-MS analysis, conventional sample extraction and concentration procedures were adapted for the investigation of the chemical composition of the volatile fraction of the collected amniotic fluid samples. In this study it was found that the amniotic fluid of Döhne Merino sheep contains very small quantities of organic compounds with polarities that vary from apolar hydrocarbons to polar carboxylic acids. Taking into consideration that the number of extraction cycles had to be limited in order to avoid the risk of losing any volatile material when a large volume of the combined extracts are concentrated for GC-MS analysis, there is no ideal solvent for the quantitative extraction of compounds with such different polarities. DCM and TBME, which are commercially available for insecticide residue and other trace analyses, were compared with respect to their ability to maximise the extraction of the amniotic fluid volatiles. TBME was subsequently selected as the most suitable solvent for this purpose. The extracts were slowly concentrated under a nitrogen atmosphere at room temperature as described in § 2.3.3.1. The resulting concentrates were used for GC-MS analyses. A typical TIC obtained in an analysis of a TBME extract of an amniotic fluid sample is shown in Fig. 3.2.

### 3.1.5 Structural elucidation of the volatile organic constituents of cranial wool and amniotic fluid of the experimental animals

During the present study the identification of the constituents of the collected natural materials was primarily based on their electron impact mass spectra (EIMS) as tentative starting point. The interpretation of the mass spectra of the majority of the volatile constituents present in the wool and the amniotic fluid samples was facilitated by initial computerised library searches using NBS and NIST libraries. The interpretation of the mass spectrum of one or two of the constituents from each compound class is discussed only very briefly. However, in the cases where the identification of some constituents proved more

challenging, the interpretation of their mass spectra and the relevant identification processes are discussed in more detail.

As far as was practically possible, the identification of constituents was confirmed by GC-MS retention time comparison with either authentic, commercially available synthetic compounds, compounds that were available from previous research projects, or compounds that were synthesised during the course of the present investigation. The synthetic compounds were made up in DCM, either individually or as mixtures containing the reference compounds in relative concentrations corresponding to those of the compounds identified in the wool or the amniotic fluid samples. Kovats retention indices (RIs) (Kovats, 1958) were determined to confirm the identification of some of the constituents under investigation. Because of the wide variation in published values, RIs were only used with discretion as additional confirmation of proposed structures.

Where appropriate, other techniques were used to determine the structures of unsaturated, branched, or chiral compounds. The application of these methods will be discussed in detail only in cases where they made a substantial contribution to the identification of the natural compounds. All relevant information is tabulated in Tables 3.1 and 3.2.

*The TICs and the mass spectra of the constituents under discussion are given at the end of this chapter in order to avoid undue fragmentation of the chapter. Only those examples of mass spectra that are required to prove a point are included in the discussions below, regardless of whether the spectrum was obtained from a constituent of the wool or an amniotic fluid sample. Both the apolar column A and the polar column B were used in GC-MS analyses to obtain mass spectral information for the identification of the volatile constituents under discussion. In the following discussion the peaks in the TICs of the wool headspace volatiles (Fig. 3.12) and amniotic fluid volatiles (Fig. 3.13) are numbered from C1*

to **C124**, and **CP1** to **CP10**<sup>6</sup>, and these numbers are used when reference is made to a constituent under discussion.

### 3.1.5.1 Saturated hydrocarbons

Constituents **C10** (Fig. 3.14), **C20**, **C28**, **C36** (Fig. 3.15), **C46**, **C58**, **C72**, **C80**, **C89** and **C100** are all present in the wool and the amniotic fluid samples, except for **C46** and **C89**, which are not present in the amniotic fluid. The EI mass spectra of these ten constituents all display a series of fragmentation ions at  $m/z$  43, 57, 71, 85, ..., characteristic of acyclic, unbranched<sup>7</sup>, saturated aliphatic hydrocarbons (Pavia *et al.*, 2001: 405–408). For example, constituent **C10** (Fig. 3.14), although present in a low concentration in the wool and amniotic fluid samples, exhibits this typical fragmentation pattern in its mass spectrum. The mass spectra of alkanes are characterised by relatively abundant molecular ions, clusters of ions occurring with gradually decreasing abundance, and the most abundant ion of each group corresponding to the general formula ( $C_nH_{2n+1}$ ), *i.e.* 14 mass units apart (as in the series above). The presence of a molecular ion at  $m/z$  128 in the EI mass spectrum of **C10** indicates that it is nonane. This identification was confirmed by retention time comparison with an authentic sample of nonane.

Constituent **C36** (Fig. 3.15) displays the same series of ions, but this constituent has no ions beyond  $m/z$  98. However, the RI of this constituent and retention time comparison with unsaturated unbranched hydrocarbons confirmed that this constituent is dodecane. Constituents **C20**, **C28**, **C46**, **C58**, **C72**, **C80**, **C89** and **C100** were likewise identified as decane, undecane, tridecane, tetradecane, hexadecane, heptadecane, octadecane and nonadecane.

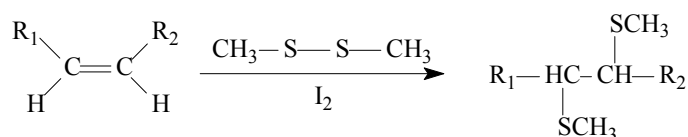
---

<sup>6</sup> Numbering of compounds: In Table 3.1 compounds identified in the amniotic fluid and wool samples are grouped according to compound classes. However, numbers were assigned from **1** to **124** according to their order of elution from Column A (apolar column), with **C** denoting “constituent”. A compound has the same number irrespective of whether it was identified in an amniotic fluid or a wool sample. Additional compounds were identified using the polar Column B (FFAP). These compounds are listed in order of elution from column B from **CP1** to **CP10**. These numbers do not imply any chemical relationship with the previous set of numbers, *e.g.* compound **C1** is 3-methylpentanal and **CP1** is 3-methyl-1-butanol.

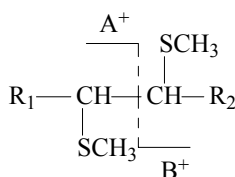
<sup>7</sup> The term “unbranched”, which is used by Rigaudy and Klesney in *Nomenclature of Organic Chemistry*, IUPAC, 1979, p. 5, is preferred to “straight-chain”.

### 3.1.5.2 Unsaturated hydrocarbons

Alkenes show distinct molecular ions because the double bonds of alkenes are capable of absorbing substantial energy. The most abundant ions in the spectra of alkenes correspond to the general formulae  $(C_nH_{2n})^+$  and  $(C_nH_{2n-1})^+$ . The mass spectra of constituents **C44** (Fig. 3.16), **C55** and **C66**, present in the wool samples, as well as the mass spectra of constituents **C118** and **C120** (Fig. 3.17), present in the wool and amniotic fluid samples, exhibit the typical fragmentation patterns of alkenes and hence these constituents were tentatively identified by computerised searches of mass spectra libraries (NBS, NIST) as 1-tridecene, 1-tetradecene, 1-pentadecene, 1-pentacosene and 1-hexacosene, respectively. The position of the double bonds in these alkenes was determined by iodine-catalysed derivatisation with DMDS, and GC-MS analysis of the derivatives (Vincenti *et al.*, 1987). The iodine-catalysed addition of DMDS can be formulated as follows:

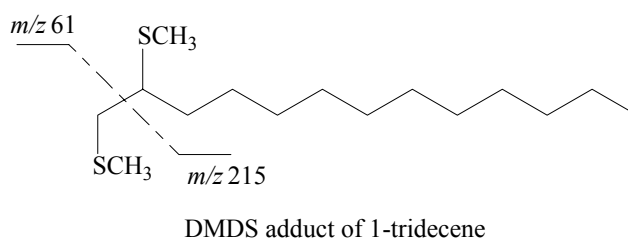


The derivatised product has a molecular ion with a mass corresponding to  $(M + 94)^+$ , as well as significantly abundant ions  $A^+$  and  $B^+$ , formed by cleavage of the adduct between the two methylthio groups (Buser *et al.*, 1983):



The position of the double bond can be deduced from these two diagnostic ions. The  $A^+$  ion belongs to the series  $m/z$  61, 75, 89, ... and the masses of the  $B^+$  series of ions depend on the structure of the functional group. The diagnostic ions are usually present in relative abundances of 30 to 100% (Burger, 2005b).

The double bond positions in constituents **C44**, **C55** and **C66** were determined without any difficulty by DMDS derivatisation (§ 3.1.2.5), even though the constituents were present in the wool samples in low concentrations. The mono-derivatised alkenes were located in the TICs by plotting single ion chromatograms for the molecular ions of the derivatives and the ions  $m/z$  215, 229 and 243, corresponding to diagnostic ions of the DMDS adducts of 1-tridecene (Fig. 3.18), 1-tetradecene and 1-pentadecene, respectively. The presence of the diagnostic ions in the mass spectrum of the DMDS adduct of, for example 1-tridecene, can be rationalised as follows:



However, the typical diagnostic ions of the DMDS derivatives of **C118** and **C120** could not be found in the TICs, possibly because the derivatives were overlapped by other major constituents of the samples. The molecular masses of the derivatives are also quite high, and it is possible that they decomposed on the column before they could be eluted, as discussed in § 3.1.2.5. This derivatisation technique is apparently not particularly suitable for the location of double bonds in high-boiling compounds (Burger, 2005b). Retention time comparison with authentic reference substances confirmed the identification of constituents **C44**, **C55**, **C66**, **C118** and **C120** as 1-tridecene, 1-tetradecene, 1-pentadecene, 1-pentacosene and 1-hexacosene, respectively. Further information confirming the structures of these 1-alkenes is given in Tables 3.1 and 3.2.

### 3.1.5.3 Unbranched primary alcohols

The EI mass spectrum of constituent **C25** (Fig. 3.19) present in the wool and amniotic fluid samples contains two prominent series of ions at  $m/z$  41, 55, 69, ... and  $m/z$  43, 57, 71, ..., with the relative abundance of the ions decreasing with increasing  $m/z$  ratio. The  $m/z$  values of these ions correspond to the general formulae  $(C_nH_{2n-1})^+$  and  $(C_nH_{2n+1})^+$ ,



respectively, and are generally observed in the mass spectra of unbranched 1-alkenes, primary alcohols and formates. The mass spectra of long-chain primary alcohols and formates closely resemble those of the corresponding 1-alkenes. Mass spectrometry should therefore be used in conjunction with other diagnostic techniques to distinguish between members of these compound classes.

Since the small but significant ion at  $m/z$  102, which is characteristic of formates, is not present in the above-mentioned mass spectrum, it seemed unlikely that this constituent could be a formate (Le Roux, 1980: 112). The molecular ions of primary and secondary alcohols are usually of very low abundance and, in the case of tertiary alcohols, they are mostly not detected. The most important fragmentation of alcohols is  $\alpha$ -cleavage with the loss of an alkyl group or the loss of  $H_2O$  (McLafferty and Tureček, 1993: 240–242). In the EI mass spectrum of **C25** a weak ion is present at  $m/z$  112. If this ion is formed by the loss of water,  $(M - 18)^+$ , then the molecular mass of **C25** should be 130, corresponding to the molecular mass of 1-octanol. Synthetic primary alcohols were used for retention time comparison and **C25** was indeed identified as 1-octanol. Constituents **C2**, **C15**, **C33**, **CP10**, **C75**, **C87** and **C98**, all present in the wool samples, were likewise identified as 1-pentanol, 1-heptanol, 1-nonanol, 1-tridecanol, 1-tetradecanol, 1-pentadecanol and 1-hexadecanol, respectively.

The mass spectra of constituents **C108**, **C114** (Fig. 3.20) and **C116** present in the wool and amniotic fluid samples contained practically no relevant diagnostic information. However, by commencing with using information obtained by mass spectra library searches and then using the methods mentioned above, these constituents were identified as 1-octadecanol, 1-eicosanol and 1-heneicosanol, respectively.

#### 3.1.5.4 Branched primary alcohols

Constituents **CP1**, present in the wool, and **C6**, present in both wool and amniotic fluid samples, were compared with published data and tentatively identified as 3-methyl-1-butanol and 4-methyl-1-pentanol, respectively. In the EI mass spectrum of constituent **C6**

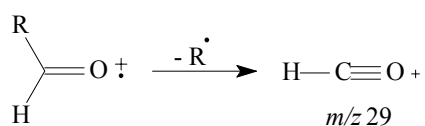
(Fig. 3.21) the base peak at  $m/z$  56 is explained by the loss of  $(\text{H}_2\text{O} + \text{C}_2\text{H}_4)$  from the molecular ion. This fragmentation process is typical for alcohols (Budzikiewicz *et al.*, 1967: 98–99). Retention time comparison with an authentic standard confirmed the identity of **C6** as 4-methyl-1-pentanol. **CP1** was similarly identified as 3-methyl-1-butanol.

### 3.1.5.5 Unbranched aldehydes

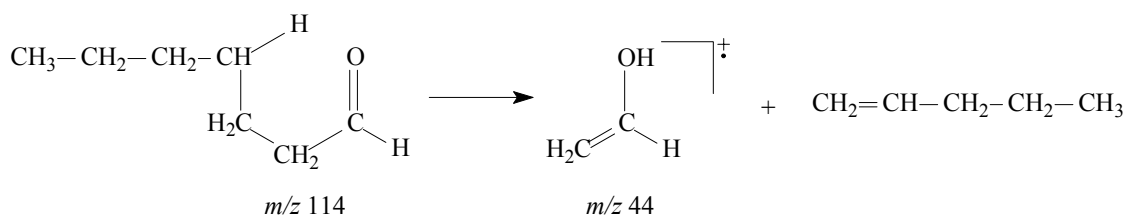
The interpretation of the mass spectra of long-chain aliphatic compounds was complicated by the paucity of diagnostic information in the higher mass ranges of the spectra of compounds present in very low concentrations in the samples under investigation. Somewhat more attention will therefore be devoted here to a discussion of the mass spectra of aldehydes, which are one of the largest groups of the wool volatiles.

The EI-mass spectra of constituents **C4**, **C8**, **C19**, **C27**, **C35**, **C45**, **C57**, **C67**, **C73**, **C81**, **C90**, **C101** and **C105**, present in both the wool and amniotic fluid samples, were compared with published data and these constituents were tentatively identified as the saturated aliphatic  $\text{C}_6$  to  $\text{C}_{18}$  aldehydes. Information on structural interpretation of mass spectra by Budzikiewicz *et al.* (1967: 130–133), McLafferty and Tureček (1993: 246–250) and Silverstein (1991: 26) was used to interpret the mass spectra of these compounds. Based on the general appearance of their mass spectra, two groups of aldehydes could be distinguished in the wool and amniotic fluid samples; the first group consisted of **C4** and **C8** and the second group consisted of the higher mass homologues.

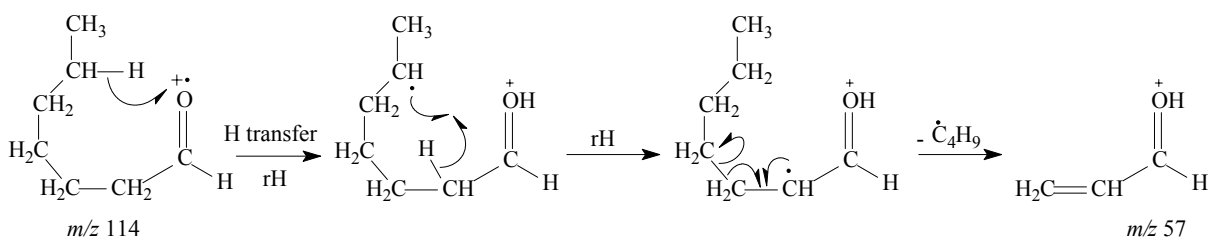
The mass spectrum of constituent **C8** (Fig. 3.22), present in all of the wool samples and in 47% of the amniotic fluid samples, can be used as an example of interpretation of the mass spectra of the lower mass homologues.  $\alpha$ -Cleavage resulting in an ion at  $m/z$  29 is observed in the spectra of short-chain aldehydes:



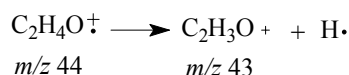
The abundant ion in the spectrum of **C8** at  $m/z$  44 is indicative of a saturated aliphatic aldehyde containing less than eight carbon atoms (Budzikiewicz *et al.*, 1967: 131). This ion can be attributed to the characteristic McLafferty rearrangement, resulting in the elimination of an olefin. In the case of heptanal the reaction can be formulated as follows:



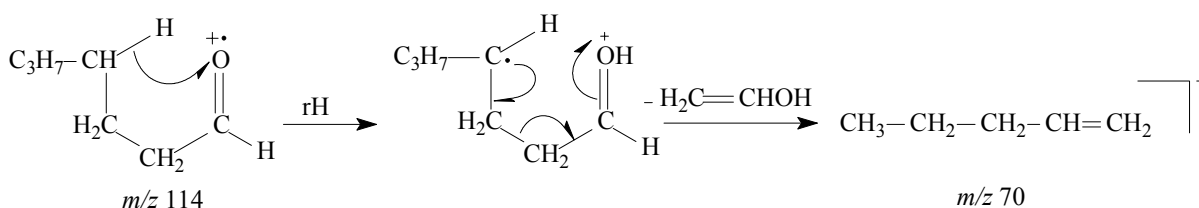
An accompanying (McLafferty + 13)<sup>+</sup> ion at  $m/z$  57 is formed in the following manner:



The oxygen-containing ion ( $\text{C}_2\text{H}_3\text{O}$ )<sup>+</sup> at  $m/z$  43 is another prominent ion in this spectrum. Evidence exists that this ion is formed as a result of the loss of a hydrogen atom from the ion at  $m/z$  44 (Harrison, 1970):



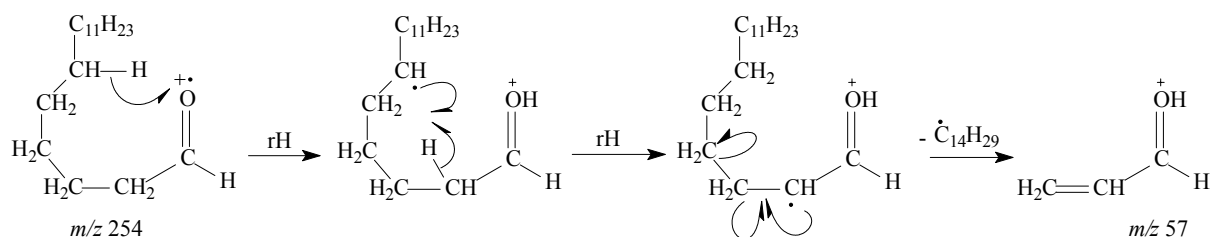
The base peak,  $(\text{M} - 44)^+$ , which in this spectrum appears at  $m/z$  70, is a characteristic ion in the mass spectra of  $\alpha$ -unsubstituted saturated aliphatic aldehydes. Sharkey *et al.* (1956) performed labelling experiments that showed that this ion ( $\text{C}_n\text{H}_{2n}$ )<sup>+</sup> is formed by the loss of  $\text{C}_2\text{H}_4\text{O}$  from the molecular ion. This implies  $\beta$ -cleavage with hydrogen transfer as in a McLafferty rearrangement, but with charge retention on the hydrocarbon fragment:



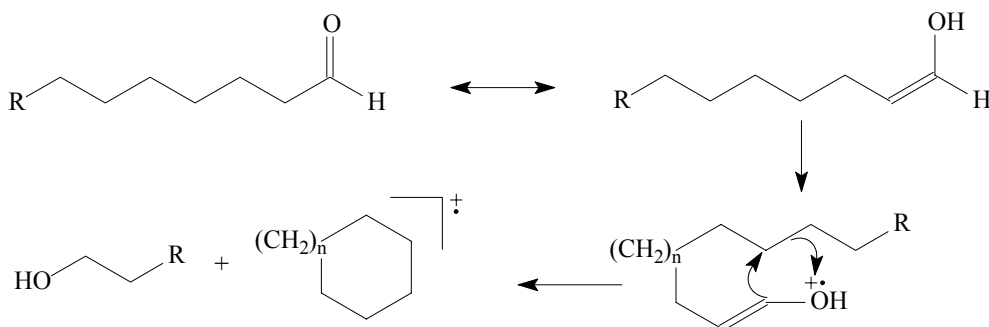
If the ion at  $m/z$  114 is accepted as the molecular ion of **C8** then the formation of the ion  $m/z$  86 can be ascribed to the loss of an ethylene molecule from the molecular ion, and the ion at  $m/z$  96 to the loss of a water molecule. The molecular mass of **C8** was determined as 114.1031 by GC-HRMS (calculated for  $\text{C}_7\text{H}_{14}\text{O}$ : 114.1045). This evidence left little doubt that **C8** is heptanal.

The interpretation of the mass spectra of the second group of higher homologues was more problematic. In the lower mass range the EI mass spectra of constituents **C19**, **C27**, **C35**, **C45**, **C57**, **C67**, **C73**, **C81**, **C90**, **C101** and **C105** are essentially the same as those of the first group. However, largely due to the very low concentrations in which some of these compounds were present in the extract, their spectra contained only a few, if any, ions in the higher mass range. It was nevertheless reasonably clear that these compounds probably are long-chain aliphatic aldehydes. However, no conclusions concerning chain branching, or molecular mass, could be reached. Constituents **C19**, **C27**, **C35**, **C45**, **C57**, **C67**, **C73**, **C81**, **C90**, **C101** and **C105** showed the regular progression of RIs expected for a homologous series.

The mass spectrum of constituent **C101** (Fig. 3.23), a member of the series mentioned above, which was present in 94% of the wool samples and in 69% of the amniotic fluid samples, will be used as an example of the interpretation of the spectra of constituents that have only a few ions in the higher mass range of their EI mass spectra. The mass spectrum of **C101** (Fig. 3.23) exhibits five prominent ions at  $m/z$  43, 57, 68, 82 and 96. The abundant ion at  $m/z$  43 is indicative of a saturated aliphatic aldehyde containing more than seven carbon atoms. The prominent (McLafferty + 13)<sup>+</sup> ion at  $m/z$  57 is formed as follows:

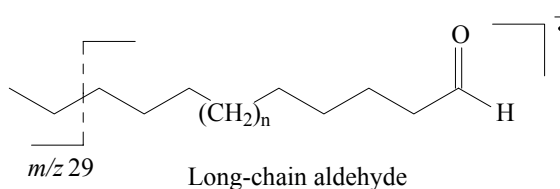


The interesting series of even-mass ions at  $m/z$  68, 82 and 96 corresponds to the general formula  $m/z (68 + 14n)^+$  ( $n = 0, 1, 2$ ). This is characteristic of the mass spectra of higher aliphatic aldehydes (Christiansen *et al.*, 1969). The elemental composition,  $(C_nH_{2n-2})^+$ , of these ions has been determined by high-resolution studies using octadecanal. From the mass spectra of a complete series of isomeric *vic*-dideutero aldehydes, Christiansen *et al.* (1969) concluded that the carbon atoms near the functional group are involved in the formation of these ions. The loss of ROH from the enol form of the aldehyde proceeds via a 4-membered ring transition state. The most abundant ion of this series occurs at  $m/z$  82, followed by less abundant ions at  $m/z$  68 and 96. This is due to the higher probability of the formation of a 6-membered ring, followed by 5- and 7-membered ring formation. The formation of the base peak at  $m/z$  82 ( $n = 1$ ), and the ions at  $m/z$  68 ( $n = 0$ ) and  $m/z$  96 ( $n = 2$ ) in the mass spectrum of **C81** can be formulated as follows (Christiansen *et al.*, 1969):



The mass spectrum of **C101** exhibits clusters of ions that are centered around  $m/z$  29, 43, 57, 85, ..., which can be ascribed to the formation of  $(C_nH_{2n+1}CO)^+$  and  $(C_nH_{2n+1})^+$  ions. As the chain length increases, this pattern becomes dominant (Silverstein *et al.*, 1991: 26).  $\alpha$ -Cleavage, resulting in an ion at  $m/z$  29, is observed in the spectra of the short-chain

aldehydes, namely formaldehyde, acetaldehyde and propionaldehyde. However, in the case of longer-chain aldehydes with higher molecular mass the ion at  $m/z$  29 is attributed to the formation of a  $C_2H_5^+$  ion from the methyl-terminal end of the carbon chain (Budzikiewicz *et al.*, 1967: 130):



The McLafferty rearrangement ion at  $m/z$  44 is present in low abundance in the mass spectrum of **C101**. There are no other diagnostic ions present in the mass spectrum and retention time comparison with a series of synthetic aldehydes confirmed the identity of this constituent as heptadecanal. Using a similar approach, **C19**, **C27**, **C35**, **C45**, **C57**, **C67**, **C73**, **C81**, **C90** and **C105** were identified as octanal, nonanal, decanal, undecanal, dodecanal, tridecanal, tetradecanal, pentadecanal, hexadecanal and Octadecanal, respectively.

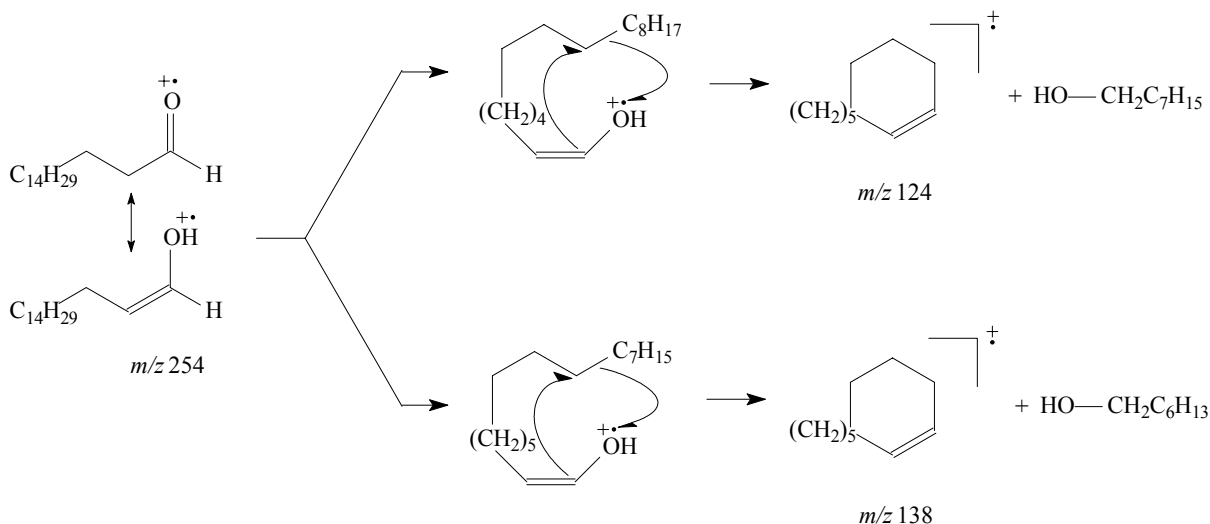
### 3.1.5.6 Branched aldehydes

Constituents **C1**, **C24**, **C31**, **C41**, **C51**, **C68**, **C77**, **C86** and **C97** are all present in the wool samples, but only **C77** and **C97** are present in the amniotic fluid samples. The constituents **C24**, **C31**, **C41**, **C51**, **C68**, **C77**, **C86** and **C97** have the regular progression of RIs expected for a homologous series. With regard to the lower mass range, the EI mass spectra of these constituents are essentially the same as those of the group of aldehydes discussed in § 3.1.5.5. However, due to the low concentrations in which the majority of these compounds are present in the wool and the amniotic fluid samples, their spectra contain only a few, if any, ions in the higher mass range. It was reasonably clear that these compounds are probably long-chain aliphatic aldehydes. The relatively high abundance of the ion at  $m/z$  43 in the spectra of these constituents suggests that they could be the iso-branched aldehydes, given that fragmentation  $\alpha$  to the methyl branching would result in the formation of  $[(CH_3)_2CH]^+$ .

It was relatively easy to identify constituent **C1** (Fig. 3.24) as 3-methylpentanal after comparing its EI mass spectrum with published data. This identification was confirmed by retention time comparison.

Constituents **C24**, **C31**, **C41**, **C51**, **C68**, **C77**, **C86** and **C97** elute from between 2.08 and 2.7 minutes earlier than the corresponding  $C_9$  to  $C_{15}$  unbranched aldehydes. The EI mass spectrum of constituent **C97**, present in 97% of the wool and in 84% of the amniotic fluid samples, are in a slightly higher concentration than the other constituents, will be used as an example of the interpretation of the spectra of this group of constituents.

The mass spectrum of **C97** (Fig. 3.25) displays ions only up to the ion cluster at  $m/z$  136, 137 and 138. The lower mass range of this spectrum displays the series of even-mass ions at  $m/z$  68, 82 and 96, corresponding to the general formula  $m/z (68 + 14n)^+$  ( $n = 0, 1, 2$ ), as discussed in § 3.1.5.5. With  $n = 4$  and  $n = 5$ , the formation of the ions present at  $m/z$  124 and  $m/z$  138 in the spectrum of **C97** can be formulated as follows:



The possible presence of iso- or anteiso-branching in constituent **C97** was considered. The isomers 14-methylhexadecanal and 15-methylhexadecanal were synthesised for comparison purposes by microscale reduction of the commercially available corresponding branched-chain carboxylic acids followed by PCC oxidation of the resulting alcohols to the

respective aldehydes (§ 2.9.5). The retention time and RI of the synthetic 15-methylhexadecanal corresponded to those of **C97**, whereas 14-methylhexadecanal eluted later than the iso-analogue. Constituents **C24**, **C31**, **C41**, **C51**, **C68**, **C77** and **C86** were similarly identified as 7-methyloctanal, 8-methylnonanal, 9-methyldecanal, 10-methylundecanal, 12-methyltridecanal, 13-methyltetra-decanal and 14-methylpentadecanal, respectively.

#### 3.1.5.7 Unsaturated aldehydes

Constituents **C23**, **C30**, **C40**, **C50**, **C62** and **C78**, all of which are present in the wool samples, were tentatively identified as  $\alpha,\beta$ -unsaturated aldehydes. The EI mass spectra of these constituents all have prominent ions at  $m/z$  41, 55, 57, 70, 83 and 97. All the constituents were present in low concentrations, and mostly display ions only up to  $m/z$  111. The mass spectra of  $\alpha,\beta$ -unsaturated aldehydes are unique, leaving little doubt as to the position of their double bonds. The double bond position of the undecenal (**50**, Fig. 3.26) was nevertheless confirmed by the presence of the diagnostic ions  $m/z$  89 and  $m/z$  173 in the mass spectrum of its DMDS derivative (Fig. 3.27). The derivatives of the other unsaturated aldehydes could not be found in the TIC of a wool extract that had been subjected to DMDS derivatisation. However, their presence in wool samples and their structures were confirmed by retention time comparison with a series of (*E*)-2-alkenals.

#### 3.1.5.8 Aromatic aldehydes

Aromatic aldehydes exhibit molecular ions of high abundance, and since the loss of a hydrogen atom from the molecular ion is energetically favourable, the formed  $(M - 1)^+$  ion is also prominent in the spectra of these compounds (Pavia *et al.*, 2001:424). The ions at  $m/z$  105 and 106 in the spectrum of constituent **C13** (Fig. 3.28) are almost of identical abundance, indicating that this constituent could be benzaldehyde (Budzikiewicz *et al.*, 1967: 162). Constituent **C22** (Fig. 3.29) displays two prominent ions in its EI mass spectrum at  $m/z$  91 (base peak) and  $m/z$  120. The  $(M - 1)^+$  ion is not prominent in this spectrum due to the lack of stabilisation of the charge on the ion by conjugation with the aromatic ring system, as in



benzaldehyde. These constituents, which are both present in the wool samples only, were identified as benzaldehyde and phenylacetaldehyde, respectively, in the usual manner.

#### 3.1.5.9 Methyl ketones

Constituents **C17**, **C26**, **C34**, **C43**, **C54**, **C65**, **C71**, **C79** and **C99** are all present in the wool samples, but only **C79** is also present in the amniotic fluid samples. The EI mass spectra of these constituents all display very prominent base peaks at  $m/z$  58. The constituents were tentatively identified as unbranched methyl ketones 2-alkanones. The mass spectra of ketones generally have relatively abundant molecular ions. According to GC-HRMS analyses the elemental composition of these ions conform to the general formula  $C_nH_{2n}O$  (Table 3.2).

The EI mass spectrum of constituent **C34** (Fig. 3.30) contains prominent ions at  $m/z$  43 and  $m/z$  58 (base peak). This is characteristic of for example, methyl ketones (NBS, NIST) and the latter ion is formed by the well-known McLafferty rearrangement. The ion present in low abundance at  $m/z$  156 could possibly be the molecular ion. Based on a mass spectra library search, **C34** was tentatively identified as 2-decanone. The base peak in the EI spectrum of **C79** (Fig. 3.31) also appears at  $m/z$  58. However, **C79** was present in low concentrations, resulting in weak EI mass spectra, and no characteristic ions are present beyond  $m/z$  111. A computerised library search suggested 2-heptadecanone as a possibility. Since constituent **C79** elutes almost a minute earlier than pentadecanal (**C81**) it could not be 2-heptadecanone. 2-Pentadecanone, prepared as described in § 2.9.6, co-eluted with the natural compound. Constituents **C17**, **C26**, **C34**, **C43**, **C54**, **C65**, **C71**, **C79** and **C99** were identified as 2-octanone, 2-nonane, 2-decanone, 2-undecanone, 2-dodecanone, 2-tridecanone, 2-tetradecanone, 2-pentadecanone and 2-heptadecanone, respectively, by employing the usual procedures (Table 3.1 and 3.2).

#### 3.1.5.10 Branched methyl ketones

The EI mass spectrum of constituent **C14** (Fig. 3.32), present in the wool and amniotic fluid samples, contains prominent ions at  $m/z$  43 (base peak) and at  $m/z$  58. A

minor ion at  $m/z$  128 could possibly be the molecular ion. Based on a mass spectra library search, **C14** was tentatively identified as 6-methyl-2-heptanone. Fragmentation  $\alpha$  to the methyl branching contributes to the high abundance of the  $m/z$  43 ion (HRMS for  $(\text{CH}_3\text{CO})^+$  calculated 43.0184, measured 43.0185). Retention time comparison with commercially available synthetic 6-methyl-2-heptanone was carried out and **C14** was identified as 6-methyl-2-heptanone.

Constituent **C47** (Fig. 3.33) was present in low concentrations in wool and amniotic fluid samples. Its EI mass spectrum contains prominent ions at  $m/z$  43 (base peak) and  $m/z$  72, but displays no ions beyond  $m/z$  97. The prominent ion at  $m/z$  43 could indicate that this constituent is a methyl ketone (Budzikiewicz *et al.*, 1967: 134), but the absence of a prominent ion at  $m/z$  58 and the presence of a base peak at  $m/z$  72 can also be construed as evidence that this constituent is either an ethyl ketone or a 3-methyl-2-alkanone. Retention time comparisons with a series of commercially available methyl and ethyl ketones showed that **C47** is neither a methyl ketone nor an ethyl ketone. However, retention-time and mass-spectral comparison with a series of 3-methyl-2-alkanones that was available from a previous research project (Burger *et al.*, 2008) strongly suggested that it could be 3-methyl-2-undecanone.

According to Burger *et al.* (2008) the *R*-enantiomers of 3-methyl-2-octanone, 3-methyl-2-nonanone and 3-methyl-2-decanone elute before the respective *S*-enantiomers from column C. The enantiomers of 3-methyl-2-undecanone were separated with a resolution  $R_S$  of 1.34 (separation factor of  $\alpha = 1.005$ ) on column C and the ratio in which the enantiomers are present was determined using the base peak of the constituent at  $m/z$  72. This branched methyl ketone is present in the wool as a practically pure *R*-enantiomer (Table 3.3).

#### 3.1.5.11 Ethyl ketones

Constituents **C16** (Fig. 3.34) and **CP4** (Fig. 3.35) were present in the wool and amniotic fluid samples in such low concentrations that their mass spectra did not contain any characteristic ions beyond  $m/z$  99 and  $m/z$  83, respectively. Computerised library searches suggested 5-methyl-3-heptanone and 3-tridecanone as possible structures for these

constituents. However, they were finally identified as 3-octanone and 3-decanone, respectively, in the usual manner.

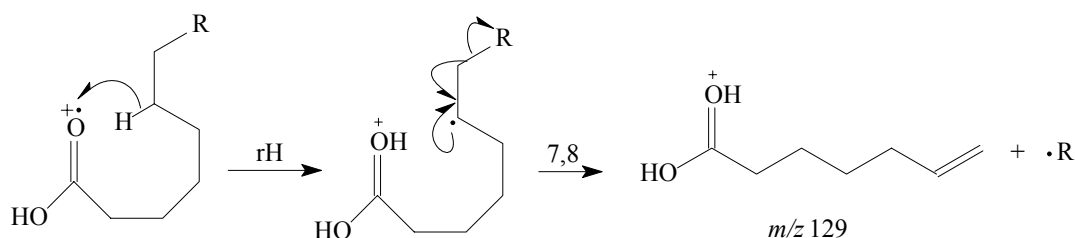
### 3.1.5.12 Unbranched carboxylic acids

In LECUS, for various reasons, carboxylic acids are subjected to GC and GC-MS analyses in their underivatised states. The broad and skewed (preailing) peak shapes with which especially the lower carboxylic acids elute from the workhorse apolar columns is compensated for by also running analyses also on columns from which such acids elute with better peak shapes, such as OV-1701 or FFAP. Constituent **CP3** (Fig. 3.36), present in the wool and amniotic fluid samples, and constituent **CP5** (Fig. 3.37), present only in the wool samples, were tentatively identified as acetic acid and propanoic acid. The mass spectrum of **CP3** displays prominent ions at  $m/z$  43 (base peak), 45 and 60 (molecular ion). The mass spectrum of **CP5** displays prominent ions at  $m/z$  28, 45 and 74 (base peak and molecular ion). These two constituents were identified as acetic acid and propanoic acid, respectively, by retention time comparison with authentic synthetic reference compounds.

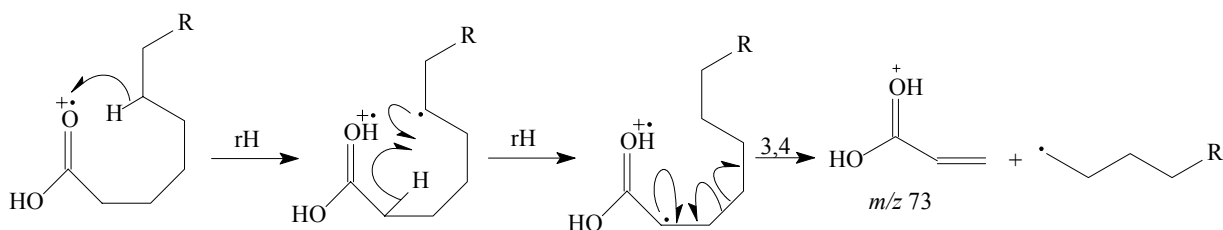
In the mass spectra of saturated short-chain aliphatic carboxylic acids the loss of OH and COOH through  $\alpha$ -cleavage on either side of the carbonyl group may be observed. The principal pathway for fragmentation of acids containing a  $\gamma$ -hydrogen atom is a McLafferty rearrangement, producing a prominent ion at  $m/z$  60 (Pavia *et al.*, 2001: 431–432). These acids generally show weak but observable molecular ions, with the abundance of the molecular ions increasing with increasing molecular mass of acids containing more than six carbon atoms (Budzikiewicz *et al.*, 1967: 214-215). The EI mass spectra of constituents **CP6**, **CP7**, **CP8**, **CP9** and **C42** (Fig. 3.38), of which only **CP6**, **CP9** and **C42** are also present in the amniotic fluid samples, are characterised by the presence of a prominent ion at  $m/z$  60. This indicates that these constituents are short-chain aliphatic acids. Retention time comparison confirmed that these constituents are butanoic acid, hexanoic acid, heptanoic acid, octanoic acid and nonanoic acid.

Constituents **C53**, **C69**, **C85** (Fig. 3.39), **C94**, **C104** (Fig. 3.40), **C107** (Fig. 3.41), **C112**, **C115**, **C117** and **C119** are present in the wool and amniotic fluid samples. The rather

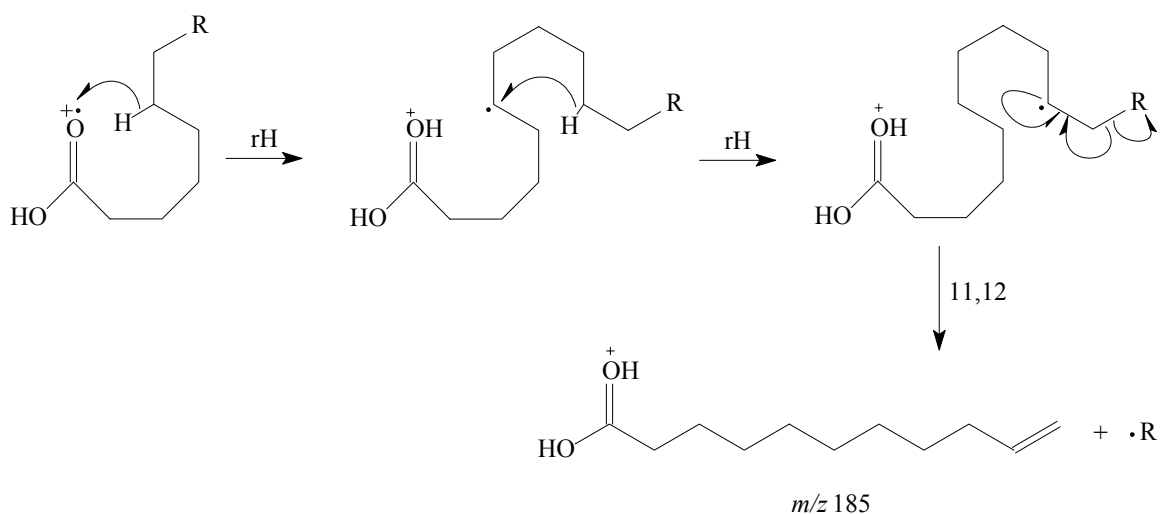
prominent ion at  $m/z$  60 and relatively abundant ions at  $m/z$  73, 129 and 185 in the mass spectra of these constituents are typically present as abundant ions in the mass spectra of carboxylic acids (Budzikiewicz *et al.*, 1967: 214–215). Although some of the constituents were present in the extracts in such low concentrations that these ions were not all present in all of their spectra. The ions at  $m/z$  73, 129 and 185 are formed by hydrogen transfer from various points along the carbon chain of the acids, in conjunction with homolysis of one of the bonds  $\alpha$  to the resulting hydrogen-depleted carbon atoms. In the cases where no carbon-to-carbon hydrogen transfer takes place, the process can be illustrated as follows (Smit, 1997: 61):



The most favoured of these reactions is the one resulting in the formation of the ion at  $m/z$  73, which is formed by the transfer of a H-atom from the  $\alpha$ -position (relative to the carbonyl group) and subsequent  $\beta$ -cleavage, as illustrated below (Spiteller, 1994: 24). The driving force for this reaction is the high stability of the  $\alpha,\beta$ -unsaturated protonated carbonyl system:



In the same manner, hydrogen migration from C-10, followed by  $\beta$ -cleavage, produces the ion at  $m/z$  185:



Many of the spectra of these constituents do not contain ions that could be considered as the molecular ions of these constituents. Retention time comparisons were carried out with a series of commercially available synthetic carboxylic acids and these constituents were subsequently identified as decanoic acid, dodecanoic acid, tetradecanoic acid, pentadecanoic acid, hexadecanoic acid, heptadecanoic acid, octadecanoic acid, eicosanoic acid, heneicosanoic acid and docosanoic acid. The molecular masses and molecular formulae of constituents **C42**, **C53**, **C69**, **C85**, **C94**, **C104**, **C107**, **C112**, **C115**, **C117** and **C119** were confirmed by HRMS (Table 3.2). The majority of these long-chain carboxylic acids have previously been identified in lanolin, as indicated in Table 3.1.

### 3.1.5.13 Branched carboxylic acids

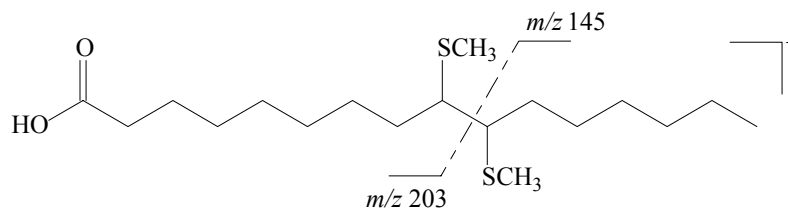
In addition to the unbranched acids discussed in the previous section, four other constituents with EI mass spectra typical of saturated long-chain aliphatic acids are present in the wool and the amniotic fluid samples. These constituents have shorter GC retention times than their unbranched counterparts and therefore they were thought to be branched aliphatic acids. Their RIs indicate that they are probably members of a homologous series. The EI mass spectra of constituents **C82** (Fig. 3.42), **C102** (Fig. 3.43), **C106** (Fig. 3.44) and **C113** are practically identical to those of tetradecanoic acid, hexadecanoic acid, heptadecanoic acid and nonadecanoic acid, respectively. However, the  $(M - 43)^+$  ion is more abundant in the spectrum of **C82**, thought to be a branched tetradecanoic acid, than in that of the

corresponding unbranched acid. This difference in abundance is smaller in the case of the hexadecanoic acids. The unbranched heptadecanoic acid was present in the samples under investigation in such a low concentration that its mass spectrum does not have any ions beyond  $m/z$  129. Although the position of branching cannot always be unequivocally deduced from the compounds' mass spectra, the similarity between the spectra of **C82**, **C102**, **C106** and **C113**, and those of the unbranched isomers, indicated that, if present, methyl branching must be far removed from the carboxyl group and hence these constituents are probably the anteiso- or isobranched carboxylic acids. Retention time comparison with authentic samples of anteiso- and isobranched long-chain carboxylic acids confirmed that constituents **C82**, **C102**, **C106** and **C113** are in fact the isobranched acids 12-methyltridecanoic acid, 14-methylpentadecanoic acid, 15-methylhexadecanoic acid and 17-methyloctadecanoic acid, respectively.

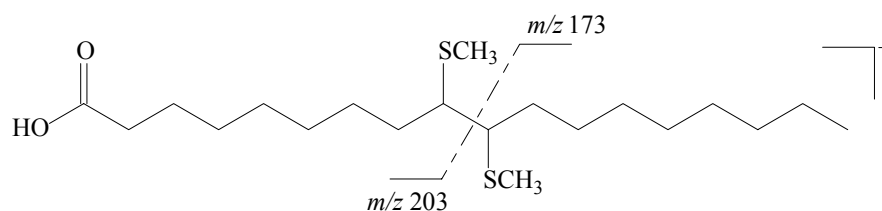
#### 3.1.5.14 Unsaturated carboxylic acids

Constituents **C103** and **C111** (Figs. 3.45 and 3.46), present in the wool and the amniotic fluid samples, have EI mass spectra in which the ions at  $m/z$  60 are present in a lower abundance than in the spectra of the saturated aliphatic acids. In addition, their spectra each contains a series of prominent ions at  $m/z$  41, 55, 69, 83, 79, ..., corresponding to the general formula  $(C_nH_{2n-1})^+$ , characteristic of unsaturation in the carbon chain (Budzikiewicz *et al.*, 1967: 55). Since **C103** elutes *ca.* 2.9 min earlier than hexadecanoic acid, and **C111** elutes 1.2 min earlier than octadecanoic acid, it was suspected that these constituents could be a hexadecenoic acid and an octadecenoic acid, respectively. The position of the double bonds in **C103** and **C111** were determined by the DMDS derivatisation technique (Vincenti *et al.*, 1987). Although the unsaturated acids did not undergo complete derivatisation, the monoderivatised acids could be located in the resulting TIC by plotting single ion chromatograms for the molecular ions of the DMDS adducts of the hexadecenoic (**C103**) and the octadecenoic acid (**C111**) at  $m/z$  348 and  $m/z$  376, respectively (Figs. 3.47 and 3.48). Having located the two DMDS derivatives in the TIC, prominent diagnostic ions were found at  $m/z$  145 and 203 and at  $m/z$  173 and 203 in the mass spectra of the hexadecenoic and octadecenoic acids, respectively. The presence of these ions in the spectra of the DMDS

derivatives can be rationalised by cleavage of the derivatives as shown below, and the constituents **C103** and **C111** are therefore (*Z*)-9-hexadecenoic acid and (*Z*)-9-octadecenoic acid, respectively.



DMDS adduct of 9-hexadecenoic acid



DMDS adduct of 9-octadecenoic acid

To confirm that there is not any branching in these two constituents, retention time comparison with authentic synthetic materials was carried out.

The EI mass spectrum of **C110** (Fig. 3.49), in the TIC of the wool and the amniotic fluid samples, was compared with published data and this constituent was tentatively identified as (*Z,Z*)-9,12-octadecadienoic acid (linoleic acid). Its DMDS adduct could not be located in the TIC, probably because the DMDS derivative decomposed on the column at its high elution temperature. A commercial reference standard was used for retention time comparison, and **C110** was identified as linoleic acid.

#### 3.1.5.15 Esters

The mass spectrum of constituent **C88** (Fig. 3.50) has a base peak at  $m/z$  88, which, in the case of ethyl esters, is formed by a McLafferty rearrangement. The ion at  $m/z$  256, which, according to high-resolution data, has the molecular composition  $C_{16}H_{32}O_2$  (Table

3.2) can therefore be tentatively accepted as the molecular ion of this constituent. Constituent **C88** co-eluted with authentic synthetic ethyl tetradecanoate, confirming the structure of this constituent.

Abundant ions are present in the EI mass spectrum of constituent **C92** (Fig. 3.51) at  $m/z$  43 (base peak), 102, 228 and 270. The latter is presumed to be the constituent's molecular ion. The EI mass spectrum of this constituent was compared with published data and the constituent was tentatively identified as isopropyl tetradecanoate. In this case the ion at  $m/z$  228,  $(M - 42)^+$ , can be ascribed to the loss of a propenyl moiety via  $\alpha$ -cleavage and the ion at  $m/z$  102 to a McLafferty rearrangement. Retention time comparison confirmed that this constituent is in fact isopropyl tetradecanoate.

#### 3.1.5.16 $\gamma$ -Lactones

The EI mass spectra of constituents **C48**, **C61**, **C76** (Fig. 3.52) and **C109** all display prominent base peaks at  $m/z$  85. The overbearing prominence of this ion, which is formed by the expulsion of the  $\gamma$ -substituent from the molecular ion, is characteristic of simple long-chain  $\gamma$ -substituted  $\gamma$ -lactones. Although these constituents were present in the extract in such low concentrations that their molecular ions are not visible in their mass spectra, the RIs of these constituents suggested that they could be unbranched  $\gamma$ -substituted  $\gamma$ -lactones. A series of  $\gamma$ -substituted  $\gamma$ -lactones available in LECUS was used for retention time comparison, and **C48**, **C61**, **C76** and **C109** were subsequently identified as nonan-4-olide, decan-4-olide, dodecan-4-olide and hexadecan-4-olide.

It is known that the *R*-enantiomers of the  $\gamma$ -lactones under discussion elute before the respective *S*-enantiomers from column C (Maas *et al.*, 1994). The enantiomers of the  $\gamma$ -lactones were separated on the above mentioned column and the base peak at  $m/z$  85 was used for quantification. The enantiomers were resolved with  $R_S$  values ranging from an average value of 1.79 for nonan-4-olide to 0.78 for dodecan-4-olide, and with separation factors ranging from an average value of  $\alpha = 1.007$  for nonan-4-olide to  $\alpha = 1.003$  for dodecan-4-olide. The enantiomers of hexadecan-4-olide (**C109**) could not be separated on



column C. Samples of amniotic fluid were subsequently analysed on column D and the enantiomers of hexadecan-4-olide were resolved with a  $R_S$  value of 0.43 and with a separation factor of  $\alpha = 1.003$ . These results are summarised in Table 3.3.

#### 3.1.5.17 Constituents with terpenoid character

Six constituents with terpenoid character were identified in the wool and amniotic fluid samples, namely **C56** (Fig. 3.53), **C60** (Fig. 3.54), **C91** (Fig. 3.55), **C93** (Fig. 3.56), **C95** (Fig. 3.57) and **C122** (Fig. 3.58). Constituents **C56** and **C60** were present in such low concentrations, and only in the SEP-enriched wool volatiles, that their mass spectra contained only a few ions that could possibly be of diagnostic value. Of the four likely structures suggested for **C56** emerging from a library search, 6,10-dimethyl-2-undecanone, otherwise known as hexahydropseudoionone, appeared to be the most likely candidate, although its similarity index was a very low 50%. Mass spectral and retention time comparison of the natural compound with 6,10-dimethyl-2-undecanone, prepared by the hydrogenation of commercially available 6,10-dimethyl-5,9-undecadien-2-one (§ 2.9.7), revealed that **C56** is in fact 6,10-dimethyl-2-undecanone.

Constituent **C60** was tentatively identified as 6,10-dimethyl-5,9-undecadien-2-one, otherwise known as dihydropseudoionone, or geranyl acetone. This structure was confirmed by the usual comparison with authentic commercially available geranyl acetone.

Constituent **C91** (Fig. 3.55) was present in very low concentrations in the headspace of the wool samples. However, its mass spectrum contains ions in the lower mass range that are typically present in the mass spectra of alkanes, albeit not throughout in the relative abundances in which they are normally present in the spectra of unbranched alkanes. The spectrum does not contain any ion that could be considered as the molecular ion of this constituent. The ion at  $m/z$  281 is due to column bleed. Since this constituent elutes later than octadecane, but earlier than nonadecane, it was thought that constituent **C91** could be a di- or higher-substituted alkane. Taking into consideration that the wool contains a few terpenoid compounds, it was assumed that **C91** could possibly be 2,6,10,14-

tetramethylhexadecane, also known as phytane. Phytane was prepared by catalytic hydrogenation of (*E,E*)-7,11,15-trimethyl-3-methylenehexadeca-1,6,10,14-tetraene ( $\beta$ -springene), which had previously been synthesised in LECUS by Le Roux (1980). Using this synthetic material and the usual spectral and retention time comparison methods this constituent was positively identified as 2,6,10,14-tetramethyl-hexadecane (Tables 3.1 and 3.2).

The mass spectrum of constituent **C93** (Fig. 3.56) contains three prominent ions at  $m/z$  43 (base peak), 58 and 71, as well as a minor ion at  $m/z$  268, which was presumed to be its molecular ion. The results of a library search indicated that this constituent could be hexahydrofarnesyl acetone (6,10,14-trimethyl-2-pentadecanone), a branched long-chain methyl ketone. The identity of this constituent, which is present in both the wool and the amniotic fluid samples, was confirmed by retention time comparison with hexahydrofarnesyl acetone, which had previously been synthesised during a previous research project (Cronje, 2006: 95–97), as well as by HRMS data (Table 3.2).

The EI mass spectrum of constituent **C95** (Fig. 3.57) which was only present in the wool samples, displays a prominent ion at  $m/z$  45, characteristic of secondary methyl carbinols (Budzikiewicz *et al.*, 1967: 96), in addition to two series of prominent ions at  $m/z$  41, 55, 69, ..., and  $m/z$  43, 57, 71, ..., corresponding to the general formulae  $C_nH_{2n-1}$  and  $C_nH_{2n+1}$ , respectively. Although a library search suggested 2-hexadecanol as the most likely candidate, **C95** elutes later than 1-pentadecanol, but earlier than 1-hexadecanol. It was assumed that **C95** contains at least 15 carbon atoms and that it also possibly has terpenoid character. Since **C93**, identified as 6,10,14-trimethyl-2-pentadecanone, elutes approximately 1.5 minutes earlier than **C95** in the TIC of the sample under investigation, the possibility was considered that this secondary alcohol could be 6,10,14-trimethyl-2-pentadecanol. Lithium aluminium hydride reduction of 6,10,14-trimethyl-2-pentadecanone, as described in § 2.9.4, yielded 6,10,14-trimethyl-2-pentadecanol, which was used to confirm the structure postulated for **C95** in the usual manner.

The EI mass spectrum of constituent **C122** (Fig. 3.58) has its base peak at  $m/z$  69 and another abundant ion at  $m/z$  81. A compound with a similar spectrum has previously been found in the dorsal secretion of the springbok (*Antidorcas marsupialis*), and was identified as (6*E*,10*E*,14*E*,18*E*)-2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene (squalene) (Le Roux, 1980: 62). Constituent **C122**, present in both the wool and amniotic fluid samples, was identified as squalene (Table 3.1 and 3.2).

### 3.1.5.18 Nitrogen containing constituents

According to library searches, constituent **C5** (Figs. 3.59) could be either a methyl-substituted pyrazine or pyrimidine, and **C11** (Fig. 3.60) either a dimethyl-substituted pyrazine or pyrimidine. These two constituents are only present in the wool samples. HRMS confirmed the molecular composition of these constituents (Table 3.2). Comparing the EI mass spectrum of **C5** (Fig. 3.59) with the spectra shown in Figs. 3.61 and 3.62, it is clear that **C5** was either methylpyrazine or 2-methylpyrimidine. These spectra are so similar that the identification of **C5** on mass spectral evidence seemed tenuous. Regarding the mass spectrum of **C11** (Fig. 3.60), at least seven candidate compounds with similar mass spectra were found in the literature, four of which are shown in Figs. 3.63 to 3.66 below:

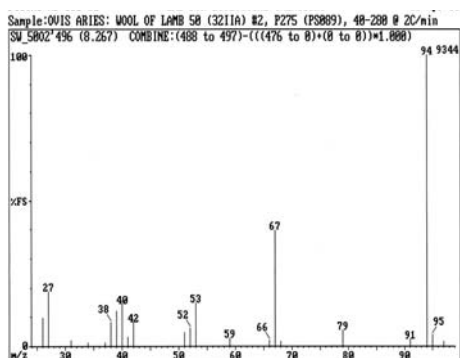


Fig. 3.59. Mass spectrum of constituent **C5**.

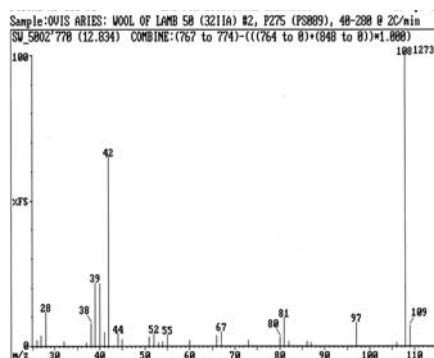


Fig. 3.60. Mass spectrum of constituent **C11**.

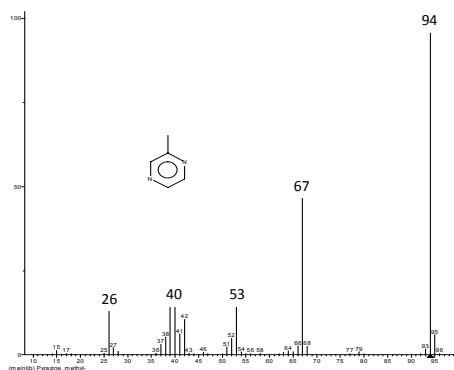


Fig. 3.61. Methylpyrazine (NIST).

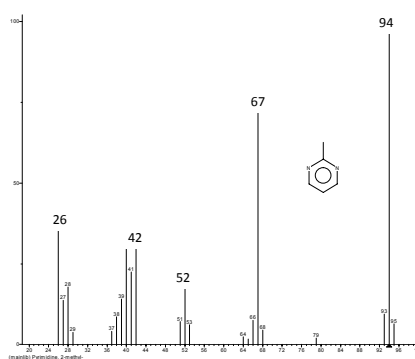


Fig. 3.62. 2-Methylpyrimidine (NIST).

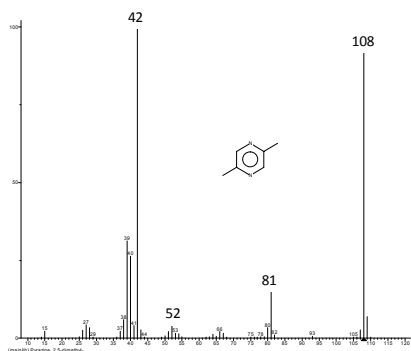


Fig. 3.63. 2,5-Dimethylpyrazine (NIST).

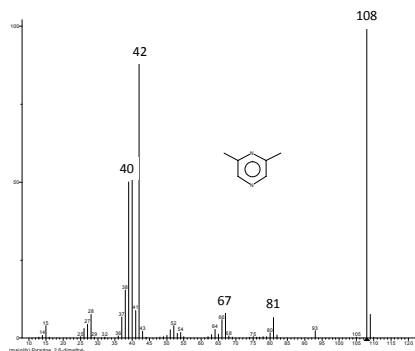


Fig. 3.64. 2,6-Dimethylpyrazine (NIST).

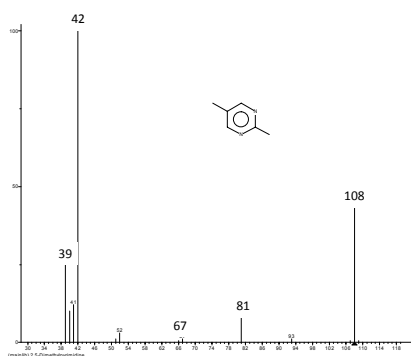


Fig. 3.65. 2,5-Dimethylpyrimidine (NIST).

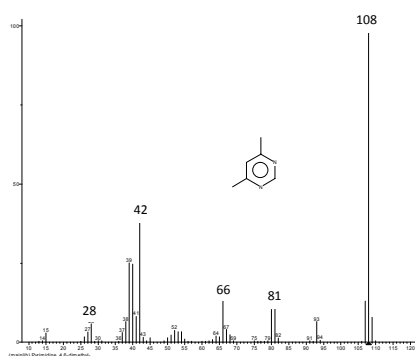


Fig. 3.66. 4,6-Dimethylpyrimidine (NIST).

Fortunately, synthetic mono- and dimethyl-substituted pyrazine and pyrimidine standards were available in LECUS for retention time comparison. Constituents **C5** and **C11** were identified as 2-methylpyrimidine and 2,5-dimethylpyrimidine, respectively.

Prominent ions are present in the EI mass spectrum of constituent **C29** (Fig. 3.67) at  $m/z$  44 and  $m/z$  113. The identification of **C29** as *N*-methyl-2-piperidinone was substantiated by HMRS data and retention time comparison with an authentic sample of the lactam (Tables 3.1 and 3.2). This constituent is only present in the wool samples.

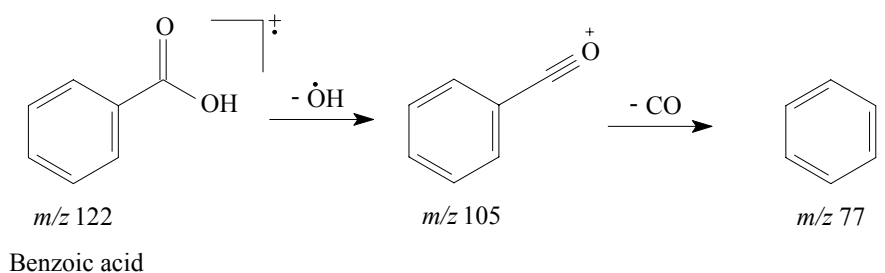
### 3.1.5.19 Miscellaneous

Constituent **CP2** (Fig. 3.68), present in low concentrations in the wool samples, displays four prominent ions in its mass spectrum at  $m/z$  55, 59 (base peak), 83 and 101, and was identified as 3-octanol.

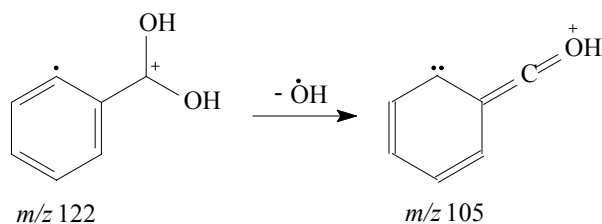
The EI mass spectrum of constituent **C9** (Fig. 3.69) displays two prominent ions: the base peak at  $m/z$  79 and an ion at  $m/z$  94. The tentative identification of this constituent as dimethyl sulphone was confirmed in the usual manner. Dimethyl sulphone is present in both the wool and the amniotic fluid samples.

The mass spectrum of constituent **C18** (Fig. 3.70), present only in the wool samples, displays a base peak at  $m/z$  81 and another prominent ion, presumed to be the molecular ion, at  $m/z$  138. Following the conventional procedure, this constituent was identified as 2-pentylfuran.

The mass spectrum of constituent **C32** (Fig. 3.71), which was identified as benzoic acid, contains prominent ions at  $m/z$  77, 105 (base peak) and 122 and the formation of ions at  $m/z$  105 and 77 can be explained in terms of simple loss of a hydroxyl radical and the carbonyl group, respectively:



However, experiments carried out with deuterated benzoic acid revealed that the hydroxyl radical eliminated in the formation of the ion at  $m/z$  105 does not consist solely of the original hydroxyl group. There are at least two forms of the molecular ion of benzoic acid which decompose at different rates (Budzikiewicz *et al.* (1967: 220). The formation of the  $m/z$  105 ion that can thus also be formulated as follows:

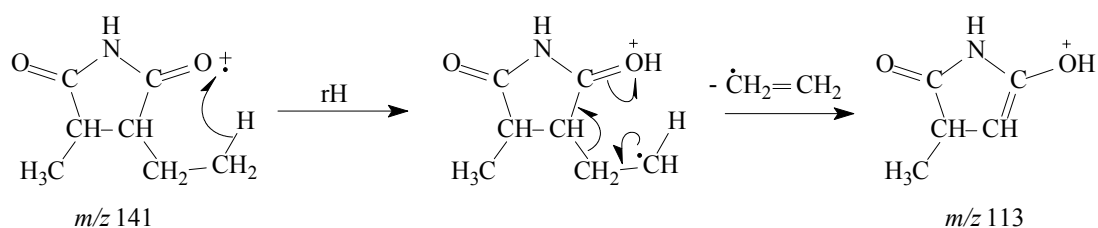


Constituent **C124** was found only in the amniotic fluid samples and is a major constituent of the amniotic fluid extracts. It elutes at *ca.* 134 min in the TICs of these samples. The ion at  $m/z$  386 in its mass spectrum (Fig. 3.72) was presumed to be the molecular ion of this constituent. The prominent ions at  $m/z$  368 and 353,  $(M - 18)^+$  and  $(M - 33)^+$ , respectively, are characteristically present in the mass spectrum of cholesterol. Constituent **C124** was identified as cholest-5-en-3 $\beta$ -ol.

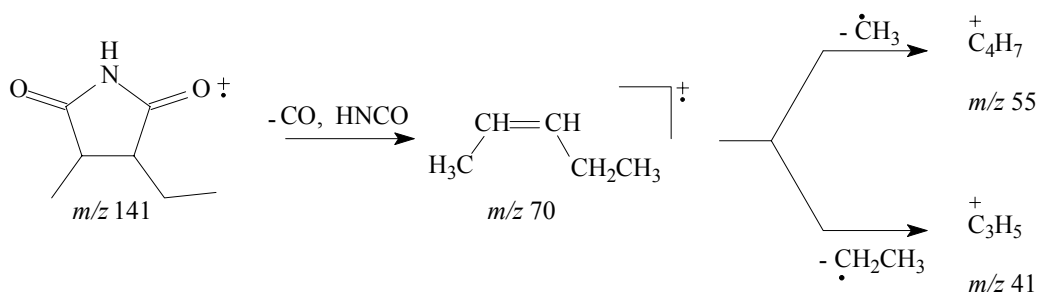
### 3.1.5.20 Tentatively identified constituents and unidentified constituents

Constituents **C37**, **C38** and **C39** (Figs. 3.73 to 3.75) are present in the wool samples, whereas only **C38** and **C39** are present in the amniotic fluid samples. Mass spectra library searches suggested 3-ethyl-4-methyl-1H-pyrrole-2,5-dione (ethylmethylmaleimide), 3-ethyl-3-methylpyrrolidine-2,5-dione (ethosuximide) and 3-methyl-4-vinyl-1H-pyrrole-2,5-dione (methylvinylmaleimide) as possible structures for constituents **C37**, **C38** and **C39**, respectively. The mass spectrum of **C38** will be discussed as an example of the mass spectra of this group of constituents. As mentioned constituent **C38** was tentatively identified as 3-ethyl-3-methylpyrrolidine-2,5-dione. However, quaternary substituted carbon atoms are rather rare in natural products, although they have been identified in, for example, the extractable organic matter of Cenomanian and Turonian black shales in Canada (Kenig *et al.*, 2005) and in the isoprenoid botryococcenes from the alga *Botryococcus braunii* (Maxwell *et*

*al.*, 1968). This led to the logical conclusion that it was more likely that **C38** could be 3-ethyl-4-methylpyrrolidine-2,5-dione. The mass spectrum of **C38** has prominent ions at  $m/z$  55 (base peak), 70 and 113. Assuming that this constituent is 3-ethyl-4-methylpyrrolidine-2,5-dione, the ion of low abundance at  $m/z$  141 has to be its molecular ion. The molecular ions of succinimide derivatives are usually weak (Sghendo *et al.*, 2002). The ion at  $m/z$  113 in the spectrum of this constituent could then be ascribed to a McLafferty rearrangement:

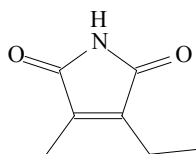


The ion at  $m/z$  70 could be ascribed to the loss of the neutral molecules CO and HNCO (Sghendo *et al.*, 2002) and the formation of the ions at  $m/z$  55 and  $m/z$  41 to the loss of either a methyl or an ethyl radical from the ion at  $m/z$  70:

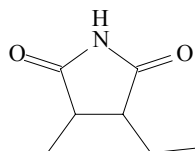


3-Ethyl-4-methyl-1H-pyrrole-2,5-dione, 3-ethyl-4-methylpyrrolidine-2,5-dione and 3-methyl-4-vinyl-1H-pyrrole-2,5-dione are not commercially available and, due to time constraints, these constituents were not synthesised for mass spectral and retention time comparison. However, it was found that the commercially available 3-ethyl-3-methylpyrrolidine-2,5-dione elutes approximately 0.58 minutes earlier than **C38**. Until reference compounds are synthesised, it will be assumed that constituents **C37**, **C38** and **C39** are 3-

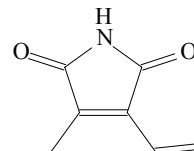
ethyl-4-methyl-1H-pyrrole-2,5-dione, 3-ethyl-4-methylpyrrolidine-2,5-dione and 3-methyl-4-vinyl-1H-pyrrole-2,5-dione, respectively (Tables 3.1 and 3.2).



3-Ethyl-4-methyl-1H-pyrrole-2,5-dione



3-Ethyl-4-methyl-pyrrolidine-2,5-dione



3-Methyl-4-vinyl-1H-pyrrole-2,5-dione

Constituent **C38** has two chiral centres and therefore four possible enantiomers. It can reasonably be accepted that the apolar column A is capable of resolving the *cis*- and *trans*-diastereomers of this compound and the presence of only one peak in TICs of wool VOCs and amniotic fluid samples indicates that only one of the diastereomers is present in the material under investigation. Therefore, the two peaks with identical mass spectra that were observed in TICs obtained on the enantioselective column C can be construed as evidence that both enantiomers of one of the diastereomers are present in a ratio of 54:46. The base peak in the enantiomers' mass spectra at  $m/z$  113 was used for quantification of the ratio in which the enantiomers are present in the amniotic fluid samples. The enantiomers were resolved on this column with a resolution,  $R_s$ , of 2.1 and a separation factor,  $\alpha$ , of 1.010 (Table 3.3). Unfortunately, the order of elution of the enantiomers of 3-ethyl-4-methylpyrrolidine-2,5-dione from column C or an equivalent column has not yet been determined. However, the possibility that column A cannot resolve the diastereomers, and column C can only resolve the diastereomers, is another possibility that cannot be totally ignored.

The mass spectrum of constituent **C96** (Fig. 3.76), present in the wool samples, has a typical mass spectrum of saturated long-chain primary alcohols. However, the RI of this constituent and retention time comparisons with primary alcohols showed that it does not belong to the series of unbranched primary alcohols, **CP10** to **C116**, discussed in § 3.1.5.3. This led to the conclusion that it could be a branched primary alcohol. It was present in very low concentrations in 88% of the 32 wool samples investigated. From its weak mass spectrum, containing only a few characteristic ions, no deductions could be made concerning



the branching of this constituent. Constituent **C96** elutes approximately 40 seconds earlier than 1-hexadecanol, and it was assumed to be a branched 1-hexadecanol. This constituent remains unidentified.

In addition to the unbranched unsaturated hydrocarbons discussed in § 3.1.5.2, constituent **C84** (Fig. 3.77) also displays a typical EI mass spectrum of a saturated long-chain alkene. However, **C84** has a lower RI than expected for an unbranched alkene and clearly does not belong to the series of 1-alkenes **C44**, **C55**, **C66**, **C118** and **C120**. Retention time comparison confirmed that **C84** is not an unbranched 1-alkene. It was present in only 25% of the investigated wool samples, but not in the amniotic fluid samples. Since it was present in low concentrations in extracts of these samples, its mass spectrum does not contain any characteristic ions beyond  $m/z$  133. Because this constituent elutes approximately 2.5 min earlier than octadecane, it was assumed that it could be a branched C18 alkene. Retention time comparison using authentic unbranched 1-alkenes confirmed that **C84** was not an unbranched 1-alkene. This constituent remains unidentified.

The mass spectra of constituents **C3** (Fig. 3.78), **C7** (Fig. 3.79), **C12** (Fig. 3.80), **C21** (Fig. 3.81), **C49** (Fig. 3.82), **C52** (Fig. 3.83), **C59** (Fig. 3.84), **C63** (Fig. 3.85), **C64** (Fig. 3.86), **C70**, (Fig. 3.87), **C74** (Fig. 3.88), **C83** (Fig. 3.89), **C121** (Fig. 3.90) and **C123** (Fig. 3.91) are either too complex and could not be to be interpreted, or contained too little information to reach meaningful conclusions. These constituents remain unidentified. Some relevant spectral information on these constituents is given in Tables 3.1 and 3.2.

### 3.1.6 Conclusions regarding the qualitative composition of the wool and the amniotic fluid samples

A total of 133 constituents were unambiguously identified in the headspace of the wool of the lambs, and 70 compounds in the amniotic fluid samples. All are listed in Table 3.1. All compounds identified in the amniotic fluid were also present in the headspace of the wool of the lambs except the unidentified steroid (**C121**), and 63 compounds were identified in the wool that were not detected in the amniotic fluid extracts. Of the 133 constituents

identified in the wool samples, 67 constituents were identified only using the apolar column A, 10 constituents were identified only using the polar column B, and 56 constituents were identified using both columns. Of the 70 compounds identified in the amniotic fluid extracts, 36 compounds were identified only using column A, three compounds only using column B, and 33 compounds using both columns A and B. A total of 65 volatile compounds not present in the amniotic fluid extracts were identified in the wool. This might be attributed to the loss of volatile molecules from the amniotic fluid extract during the extraction and concentration process (Soini *et al.*, 2005), and the greater sensitivity of the SEP enrichment technique for volatile molecules, compared to techniques relying on the extraction of volatiles from liquid phases in those cases where continuous liquid–liquid extraction is not an option.

Several compound classes are represented in the headspace of the wool and in the volatile material extracted from the amniotic fluid. The majority of these compounds have previously been identified in the excretions and secretions of numerous mammalian species (Burger *et al.*, 2005a and 2008). The aliphatic constituents extracted from these two substrates include saturated and unsaturated branched and unbranched hydrocarbons, branched and unbranched primary alcohols, a branched and an unbranched secondary alcohol, saturated branched and unbranched aldehydes, unsaturated unbranched aldehydes, aromatic aldehydes, saturated branched and unbranched methyl ketones, an unsaturated branched methyl ketone, saturated and unsaturated branched and unbranched carboxylic acids, an aromatic carboxylic acid, an ethyl- and an isopropyl ester and dimethyl sulphone. Various heterocyclic compounds were also identified, namely 2-pentylfuran, butanolides, methyl-substituted pyrimidines, *N*-methyl-2-piperidinone and cholest-5-en-3 $\beta$ -ol. Three heterocyclic compounds, 3-ethyl-4-methyl-1H-pyrrole-2,5-dione, 3-ethyl-4-methyl-pyrrolidine-2,5-dione and 3-methyl-4-vinyl-1H-pyrrole-2,5-dione were tentatively identified. A large number of the carboxylic acids, hydrocarbons and alcohols identified in this study have previously been identified in lanolin (Motiuk, 1979a, 1979b, 1980; Schlossman and McCarthy, 1979).

---

---

Except for a few minor terpenoids, the only other chiral constituents identified in the wool and in the amniotic fluid samples were 3-methyl-2-undecanone, a series of  $\gamma$ -substituted  $\gamma$ -lactones, 3-methylpentanal and 3-ethyl-4-methylpyrrolidine-2,5-dione. None of the chiral terpenoids, nor 3-methylpentanal, present in the wool and amniotic fluid samples were resolved on the chiral columns C and D. All the results obtained from the enantioselective GC-MS analyses are summarised in Table 3.3.

The majority of the compounds identified in this study are present in low concentrations in the wool and in the amniotic fluid extracts, as shown in Figs. 3.12 and 3.13. It is possible that some volatile compounds were not identified in the wool and the amniotic fluid because they were present in such low concentrations that their mass spectra do not contain useful diagnostic information.

The analytical protocols developed in 2007 were followed in every detail during the lambing season of 2009. The VOCs were identified in the wool of lambs born during 2009 to ewes that were also in the sample group of 2007. Of the 87 compounds identified in 2007, 14 compounds were not detected and 10 new compounds were identified in 2009. Of these 10 new compounds, five were also identified in the amniotic fluid collected in 2007. These results are summarised in Table 3.1.

**Table 3.1: VOCs of the amniotic fluid of Döhne Merino ewes and the cranial wool of their lambs collected during the lambing seasons of 2007 and 2009**

No.	Compound	EI-mass spectrum	Column*		Amniotic fluid	Wool	Remarks
			Apolar	Polar			
10	Nonane	Fig. 3.14	✓		✓	✓	a,c,d,l,n
20	Decane		✓		✓	✓	a,b,c,d,l,n,p
28	Undecane		✓		✓	✓	a,b,c,d,p
36	Dodecane	Fig. 3.15	✓	✓	✓	✓	a,b,c,d,p
46	Tridecane		✓			✓	a,b,c,d,k,p
58	Tetradecane		✓	✓	✓	✓	a,b,c,d,k,o,p
72	Hexadecane		✓			✓	a,b,c,d,k,p
80	Heptadecane		✓		✓	✓	a,b,c,d,k,p
89	Octadecane		✓		✓	✓	a,b,c,d,k,p
100	Nonadecane		✓			✓	a,b,c,d,k,p
44	1-Tridecene	Fig. 3.16	✓			✓	a,b,c,d,f,p
55	1-Tetradecene		✓			✓	a,b,c,d,f,n,p
66	1-Pentadecene		✓			✓	a,b,c,d,f,p
118	1-Pentacosene		✓		✓	✓	a,b,c,e
120	1-Hexacosene	Fig. 3.17	✓		✓	✓	a,b,c,e
2	1-Pentanol		✓			✓	a,c,d,l
15	1-Heptanol		✓	✓		✓	a,c,d,p
25	1-Octanol	Fig. 3.19	✓	✓	✓	✓	a,c,d,k,p
33	1-Nonanol		✓	✓		✓	a,c,d,p
P10	1-Tridecanol			✓		✓	a,c,d
75	1-Tetradecanol		✓	✓		✓	a,c,d,k,m
87	1-Pentadecanol		✓			✓	a,c,d,k,m,p
98	1-Hexadecanol		✓	✓		✓	a,c,d,k,m,n
108	1-Octadecanol		✓		✓	✓	a,c,d,k,m
114	1-Eicosanol	Fig. 3.20	✓		✓	✓	a,c,d,k
116	1-Heneicosanol		✓		✓	✓	a,c,e,k
P1	3-Methyl-1-butanol			✓		✓	a,c,d
6	4-Methyl-1-pentanol	Fig. 3.21	✓	✓	✓	✓	a,c,d
4	Hexanal		✓		✓	✓	a,c,d,l
8	Heptanal	Fig. 3.22	✓	✓	✓	✓	a,b,c,d,l,p
19	Octanal		✓	✓	✓	✓	a,b,c,d,p
27	Nonanal		✓	✓	✓	✓	a,b,c,d,p
35	Decanal		✓	✓	✓	✓	a,b,c,d,p

Table 3.1 (continued)

No.	Compound	EI-mass spectrum	Column*		Amniotic fluid	Wool	Remarks
			Apolar	Polar			
45	Undecanal		✓		✓	✓	a,c,d,p
57	Dodecanal		✓	✓	✓	✓	a,b,c,d,p
67	Tridecanal		✓	✓	✓	✓	a,b,c,d,p
73	Tetradecanal		✓	✓	✓	✓	a,c,d,p
81	Pentadecanal		✓	✓	✓	✓	a,b,c,d,p
90	Hexadecanal		✓		✓	✓	a,b,c,d,p
101	Heptadecanal	Fig. 3.23	✓		✓	✓	a,c,d,p
105	Octadecanal		✓		✓	✓	a,c,d
1	3-Methylpentanal	Fig. 3.24	✓			✓	a,c,d,h
24	7-Methyloctanal		✓			✓	a,c,d,e,n,p
31	8-Methylnonanal		✓			✓	a,c,d,e,p
41	9-Methyldecanal		✓			✓	a,b,c,d,e,p
51	10-Methylundecanal		✓	✓		✓	a,c,d,e,p
68	12-Methyltridecanal		✓			✓	a,c,d,e
77	13-Methyltetradecanal		✓		✓	✓	a,c,d,e,p
86	14-Methylpentadecanal		✓			✓	a,c,d,e,n,p
97	15-Methylhexadecanal	Fig. 3.25	✓		✓	✓	a,c,d,e,n
23	( <i>E</i> )-2-Octenal		✓	✓		✓	a,b,c,d,e,p
30	( <i>E</i> )-2-Nonenal		✓	✓		✓	a,c,d,e,p
40	( <i>E</i> )-2-Decenal		✓	✓		✓	a,b,c,d,e,p
50	( <i>E</i> )-2-Undecenal	Fig. 3.26	✓	✓		✓	a,b,c,d,e,f,p
62	( <i>E</i> )-2-Dodecenal		✓			✓	a,c,d,e,n,p
78	( <i>E</i> )-2-Tetradecenal		✓			✓	a,c,d,e
13	Benzaldehyde	Fig. 3.28	✓			✓	a,c,d,o,p
22	Phenylacetaldehyde	Fig. 3.29	✓			✓	a,c,d,o,p
17	2-Octanone		✓	✓		✓	a,b,c,d,l,p
26	2-Nonanone		✓			✓	a,b,c,d,p
34	2-Decanone	Fig. 3.30	✓	✓		✓	a,b,c,d,p
43	2-Undecanone		✓			✓	a,b,c,d,p
54	2-Dodecanone		✓	✓		✓	a,b,c,d,p
65	2-Tridecanone		✓	✓		✓	a,b,c,d,p
71	2-Tetradecanone		✓	✓		✓	a,b,c,d
79	2-Pentadecanone	Fig. 3.31	✓	✓	✓	✓	a,b,c,d,p

Table 3.1 (continued)

No.	Compound	EI-mass spectrum	Column*		Amniotic fluid	Wool	Remarks
			Apolar	Polar			
99	2-Heptadecanone		✓	✓		✓	a,b,c,d,p
14	6-Methyl-2-heptanone	Fig. 3.32	✓	✓	✓	✓	a,b,c,d,l,p
47	3-Methyl-2-undecanone	Fig. 3.33	✓		✓	✓	a,c,d,e,g
16	3-Octanone	Fig. 3.34	✓		✓	✓	a,b,c,d,p
P4	3-Decanone	Fig. 3.35		✓		✓	a,c,d
P3	Acetic acid	Fig. 3.36		✓	✓	✓	a,c,d
P5	Propanoic acid	Fig. 3.37		✓		✓	a,c,d
P6	Butanoic acid			✓	✓	✓	a,c,d
P7	Hexanoic acid			✓		✓	a,c,d
P8	Heptanoic acid			✓		✓	a,c,d
P9	Octanoic acid			✓	✓	✓	a,c,d,k
42	Nonanoic acid	Fig. 3.38	✓	✓	✓	✓	a,b,c,d,k
53	Decanoic acid		✓	✓	✓	✓	a,b,c,d,k
69	Dodecanoic acid		✓	✓	✓	✓	a,b,c,d,k,n
85	Tetradecanoic acid	Fig. 3.39	✓	✓	✓	✓	a,b,c,d,k,p
94	Pentadecanoic acid		✓	✓	✓	✓	a,b,c,d,k,o,p
104	Hexadecanoic acid	Fig. 3.40	✓	✓	✓	✓	a,b,c,d,k,n,p
107	Heptadecanoic acid	Fig. 3.41	✓		✓	✓	a,b,c,d,k
112	Octadecanoic acid		✓	✓	✓	✓	a,b,c,d,k
115	Eicosanoic acid		✓	✓	✓	✓	a,b,c,d,k
117	Heneicosanoic acid		✓		✓	✓	a,b,c,d,k
119	Docosanoic acid		✓		✓	✓	a,b,c,d,k
82	12-Methyltridecanoic acid	Fig. 3.42	✓		✓	✓	a,b,c,d,e,k
102	14-Methylpentadecanoic acid	Fig. 3.43	✓	✓	✓	✓	a,b,c,d,e,k
106	15-Methylhexadecanoic acid	Fig. 3.44	✓		✓	✓	a,b,c,d,e,k
113	17-Methyloctadecanoic acid		✓		✓	✓	a,b,c,d,e,k
103	(Z)-9-Hexadecenoic acid	Fig. 3.45	✓	✓	✓	✓	a,b,c,d,f
110	(Z,Z)-9,12-Octadecadienoic acid	Fig. 3.49	✓	✓	✓	✓	a,b,c,d
111	(Z)-9-Octadecenoic acid	Fig. 3.46	✓	✓	✓	✓	a,b,c,d,f
88	Ethyl tetradecanoate	Fig. 3.50	✓	✓		✓	a,b,c,d,p
92	Isopropyl tetradecanoate	Fig. 3.51	✓		✓	✓	a,b,c,d,e,p

Table 3.1 (continued)

No.	Compound	EI-mass spectrum	Column*		Amniotic fluid	Wool	Remarks
			Apolar	Polar			
48	Nonan-4-olide		✓	✓	✓	✓	a,b,c,d,g,p
61	Decan-4-olide		✓		✓	✓	a,b,c,d,g
76	Dodecan-4-olide	Fig. 3.52	✓		✓	✓	a,b,c,d,g
109	Hexadecan-4-olide		✓		✓	✓	a,b,c,d,g
56	6,10-Dimethyl-2-undecanone	Fig. 3.53	✓			✓	a,b,c,d,h
60	(5 <i>E</i> )-6,10-Dimethyl-5,9-undecadien-2-one	Fig. 3.54	✓			✓	a,b,c,d,p
91	2,6,10,14-Tetramethyl-hexadecane	Fig. 3.55	✓			✓	a,c,d,h
93	6,10,14-Trimethyl-2-pentadecanone	Fig. 3.56	✓	✓	✓	✓	a,b,c,d,h,p
95	6,10,14-Trimethyl-2-pentadecanol	Fig. 3.57	✓			✓	a,c,d,h
122	Squalene	Fig. 3.58	✓	✓	✓	✓	a,b,c,d,j
5	2-Methylpyrimidine	Fig. 3.59	✓	✓		✓	a,b,c,d
11	2,5-Dimethylpyrimidine	Fig. 3.60	✓	✓		✓	a,b,c,d
29	<i>N</i> -Methyl-2-piperidinone	Fig. 3.67	✓	✓		✓	a,b,c,d
p2	3-Octanol	Fig. 3.68		✓		✓	a,c,d
9	Dimethyl sulphone	Fig. 3.69	✓	✓	✓	✓	a,b,c,d,o,p
18	2-Pentylfuran	Fig. 3.70	✓			✓	a,b,c,d,p
32	Benzoic acid	Fig. 3.71	✓	✓	✓	✓	a,c,d
124	Cholest-5-en-3 $\beta$ -ol	Fig. 3.72	✓		✓	✓	a,b,c,d,k
37	3-Ethyl-4-methyl-1H-pyrrole-2,5-dione	Fig. 3.73	✓	✓		✓	a,b,c,i,o,p
38	3-Ethyl-4-methylpyrrolidine-2,5-dione	Fig. 3.74	✓	✓	✓	✓	a,b,i,g,o
39	3-Methyl-4-vinyl-1H-pyrrole-2,5-dione	Fig. 3.75	✓	✓	✓	✓	a,b,c,i,o
3	Unidentified	Fig. 3.78	✓			✓	
7	Unidentified	Fig. 3.79	✓			✓	
12	Unidentified	Fig. 3.80	✓	✓		✓	o
21	Unidentified	Fig. 3.81	✓			✓	
49	Unidentified	Fig. 3.82	✓	✓		✓	o,p
52	Unidentified	Fig. 3.83	✓			✓	n
59	Unidentified	Fig. 3.84	✓		✓	✓	
63	Unidentified	Fig. 3.85	✓	✓	✓	✓	

Table 3.1 (continued)

No.	Compound	EI-mass spectrum	Column*		Amniotic fluid	Wool	Remarks
			Apolar	Polar			
64	Unidentified	Fig. 3.86	✓			✓	p
70	Unidentified	Fig. 3.87	✓			✓	p
74	Unidentified	Fig. 3.88	✓			✓	n
83	Unidentified	Fig. 3.89	✓			✓	n
84	Unidentified	Fig. 3.77	✓			✓	a,b,n
96	Unidentified	Fig. 3.76	✓			✓	a,c,p
121	Unidentified	Fig. 3.90	✓		✓		
123	Unidentified	Fig. 3.91	✓		✓	✓	

\*Column used to identify constituent

- a: Low-resolution EI mass spectrum
- b: High-resolution EI mass spectrum; information given in Table 3.2
- c: Library spectrum (NBS and/or NIST)
- d: Retention time comparison with synthetic compound
- e: Kovats retention index
- f: Double bond localisation by DMDS derivatisation and GC-MS analysis
- g: Absolute configuration given in Table 3.3
- h: Absolute configuration not determined
- i: Tentative identification
- j: Compounds previously identified in amniotic fluid (Rietdorf, 2002)

- k: Compounds previously identified in lanolin (Schlossman and McCarthy, 1979; Motiuk, 1979a, 1979b, 1980)
- l: Compounds previously identified in wool (Lisovac and Shooter, 2003)
- m: Compounds previously identified in inguinal gland of ewes (Rietdorf, 2002)
- n: Compounds identified in the wool during the lambing season of 2007 that were not identified in the lambing season of 2009
- o: Compounds identified in the wool during the lambing season of 2009 that were not identified in the lambing season of 2007
- p: Compounds identified in cloth jackets left on lamb for 3 days



**Table 3.2: VOCs present in the amniotic fluid of Döhne Merino ewes and the cranial wool of their lambs collected during the lambing seasons of 2007 and 2009 identified with HRMS**

No.	Compound	Molecular formula	Molecular mass		Mass difference (mDa) <sup>a</sup>
			Measured	Calculated	
20	Decane	C <sub>10</sub> H <sub>22</sub>	142.1709	142.1722	-1.30
36	Dodecane	C <sub>12</sub> H <sub>26</sub>	170.2038	170.2035	0.30
28	Undecane	C <sub>11</sub> H <sub>24</sub>	156.1892	156.1878	1.40
46	Tridecane	C <sub>13</sub> H <sub>28</sub>	184.2192	184.2191	0.10
58	Tetradecane	C <sub>14</sub> H <sub>30</sub>	198.2353	198.2348	0.50
72	Hexadecane	C <sub>16</sub> H <sub>34</sub>	226.2675	226.2661	1.40
80	Heptadecane	C <sub>17</sub> H <sub>36</sub>	240.2823	240.2817	0.60
89	Octadecane	C <sub>18</sub> H <sub>38</sub>	254.2972	254.2974	-0.20
100	Nonadecane	C <sub>19</sub> H <sub>40</sub>	268.3137	268.3130	0.70
44	1-Tridecene	C <sub>13</sub> H <sub>26</sub>	182.2022	182.2035	-1.30
55	1-Tetradecene	C <sub>14</sub> H <sub>28</sub>	196.2189	196.2191	-0.20
66	1-Pentadecene	C <sub>15</sub> H <sub>30</sub>	210.2337	210.2348	-1.10
118	1-Pentacosene	C <sub>25</sub> H <sub>50</sub>	350.3914	350.3913	0.10
120	1-Hexacosene	C <sub>26</sub> H <sub>52</sub>	364.4089	364.4069	2.00
15	1-Heptanol	C <sub>7</sub> H <sub>14</sub> <sup>b</sup>	98.1081	98.1096	-1.50
25	1-Octanol	C <sub>8</sub> H <sub>16</sub> <sup>b</sup>	112.1266	112.1252	1.40
75	1-Tetradecanol	C <sub>14</sub> H <sub>28</sub> <sup>b</sup>	196.2193	196.2191	0.20
98	1-Hexadecanol	C <sub>16</sub> H <sub>32</sub> <sup>b</sup>	224.2515	224.2504	1.10
108	1-Octadecanol	C <sub>18</sub> H <sub>36</sub> <sup>b</sup>	252.2836	252.2817	1.90
114	1-Eicosanol	C <sub>20</sub> H <sub>40</sub> <sup>b</sup>	280.3150	280.3130	2.00
116	1-Heneicosanol	C <sub>21</sub> H <sub>42</sub> <sup>b</sup>	294.3281	294.3287	-0.60
8	Heptanal	C <sub>7</sub> H <sub>14</sub> O	114.1031	114.1045	-1.40
19	Octanal	C <sub>8</sub> H <sub>16</sub> O	128.1200	128.1201	-0.10
27	Nonanal	C <sub>9</sub> H <sub>18</sub> O	142.1349	142.1358	-0.90
35	Decanal	C <sub>10</sub> H <sub>20</sub> O	156.1525	156.1514	1.10
45	Undecanal	C <sub>11</sub> H <sub>22</sub> O	170.1653	170.1671	-1.80
57	Dodecanal	C <sub>12</sub> H <sub>24</sub> O	184.1825	184.1827	-0.20
67	Tridecanal	C <sub>13</sub> H <sub>26</sub> O	198.1985	198.1984	0.10
73	Tetradecanal	C <sub>14</sub> H <sub>28</sub> O	212.2154	212.2140	1.40
81	Pentadecanal	C <sub>15</sub> H <sub>30</sub> O	226.2295	226.2297	-0.20
90	Hexadecanal	C <sub>16</sub> H <sub>32</sub> O	240.2465	240.2453	1.20
101	Heptadecanal	C <sub>17</sub> H <sub>34</sub> O	254.2601	254.2610	-0.90
105	Octadecanal	C <sub>18</sub> H <sub>36</sub> O	268.2788	268.2766	2.20
24	7-Methyloctanal	C <sub>9</sub> H <sub>18</sub> O	142.1360	142.1358	0.20
41	9-Methyldecanal	C <sub>11</sub> H <sub>22</sub> O	170.1652	170.1671	-1.90
97	15-Methylhexadecanal	C <sub>17</sub> H <sub>34</sub> O	254.2606	254.2610	-0.40

Table 3.2 (continued)

No.	Compound	Molecular formula	Molecular mass		Mass difference (mDa <sup>a</sup> )
			Measured	Calculated	
23	( <i>E</i> )-2-Octenal	C <sub>8</sub> H <sub>14</sub> O	126.1058	126.1045	1.30
30	( <i>E</i> )-2-Nonenal	C <sub>9</sub> H <sub>16</sub> O	140.1214	140.1201	1.30
40	( <i>E</i> )-2-Decenal	C <sub>10</sub> H <sub>18</sub> O	154.1372	154.1358	1.40
50	( <i>E</i> )-2-Undecenal	C <sub>11</sub> H <sub>20</sub> O	168.1495	168.1514	-1.90
17	2-Octanone	C <sub>8</sub> H <sub>16</sub> O	128.1196	128.1201	-0.50
26	2-Nonanone	C <sub>9</sub> H <sub>18</sub> O	142.1368	142.1358	1.00
34	2-Decanone	C <sub>10</sub> H <sub>20</sub> O	156.1511	156.1514	-0.30
43	2-Undecanone	C <sub>11</sub> H <sub>22</sub> O	170.1682	170.1671	1.10
54	2-Dodecanone	C <sub>12</sub> H <sub>24</sub> O	184.1827	184.1827	0.00
65	2-Tridecanone	C <sub>13</sub> H <sub>26</sub> O	198.1990	198.1984	0.60
71	2-Tetradecanone	C <sub>14</sub> H <sub>28</sub> O	212.2133	212.2140	-0.70
79	2-Pentadecanone	C <sub>15</sub> H <sub>30</sub> O	226.2301	226.2297	0.40
99	2-Heptadecanone	C <sub>17</sub> H <sub>34</sub> O	254.2617	254.2610	0.70
14	6-Methyl-2-heptanone	C <sub>8</sub> H <sub>16</sub> O	128.1195	128.1201	-0.60
16	3-Octanone	C <sub>8</sub> H <sub>16</sub> O	128.1188	128.1201	-1.30
42	Nonanoic acid	C <sub>9</sub> H <sub>18</sub> O <sub>2</sub>	158.1300	158.1307	-0.70
53	Decanoic acid	C <sub>10</sub> H <sub>20</sub> O <sub>2</sub>	172.1469	172.1463	0.60
69	Dodecanoic acid	C <sub>12</sub> H <sub>24</sub> O <sub>2</sub>	200.1776	200.1776	0.00
85	Tetradecanoic acid	C <sub>14</sub> H <sub>28</sub> O <sub>2</sub>	228.2089	228.2089	0.00
94	Pentadecanoic acid	C <sub>15</sub> H <sub>30</sub> O <sub>2</sub>	242.2254	242.2246	0.80
104	Hexadecanoic acid	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	256.2413	256.2402	1.10
107	Heptadecanoic acid	C <sub>17</sub> H <sub>34</sub> O <sub>2</sub>	270.2574	270.2559	1.50
112	Octadecanoic acid	C <sub>18</sub> H <sub>36</sub> O <sub>2</sub>	284.2717	284.2715	0.20
115	Eicosanoic acid	C <sub>20</sub> H <sub>40</sub> O <sub>2</sub>	312.3041	312.3028	1.30
117	Heneicosanoic acid	C <sub>21</sub> H <sub>42</sub> O <sub>2</sub>	326.3203	326.3185	1.80
119	Docosanoic acid	C <sub>22</sub> H <sub>44</sub> O <sub>2</sub>	340.3360	340.3341	1.90
82	12-Methyltridecanoic acid	C <sub>14</sub> H <sub>28</sub> O <sub>2</sub>	228.2097	228.2089	0.80
102	14-Methylpentadecanoic acid	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	256.2404	256.2402	0.20
106	15-Methylhexadecanoic acid	C <sub>17</sub> H <sub>34</sub> O <sub>2</sub>	270.2579	270.2559	2.00
113	17-Methyloctadecanoic acid	C <sub>19</sub> H <sub>38</sub> O <sub>2</sub>	298.2900	298.2872	1.80
103	( <i>Z</i> )-9-Hexadecenoic acid	C <sub>16</sub> H <sub>30</sub> O <sub>2</sub>	254.2230	254.2246	-1.60
111	( <i>Z</i> )-9-Octadecenoic acid	C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>	282.2544	282.2559	-1.50
110	( <i>Z,Z</i> )-9,12-Octadecadienoic acid	C <sub>18</sub> H <sub>32</sub> O <sub>2</sub>	280.2422	280.2402	2.00
88	Ethyl tetradecanoate	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	256.2406	256.2402	0.40

**Table 3.2 (continued)**

No.	Compound	Molecular formula	Molecular mass		Mass difference (mDa) <sup>a</sup>
			Measured	Calculated	
92	Isopropyl tetradecanoate	C <sub>17</sub> H <sub>34</sub> O <sub>2</sub>	270.2563	270.2559	0.40
48	Nonan-4-olide	C <sub>9</sub> H <sub>16</sub> O <sub>2</sub>	156.1145	156.1150	-0.50
61	Decan-4-olide	C <sub>10</sub> H <sub>18</sub> O <sub>2</sub>	170.1303	170.1307	-0.40
76	Dodecan-4-olide	C <sub>12</sub> H <sub>22</sub> O <sub>2</sub>	198.1626	198.1620	0.60
109	Hexadecan-4-olide	C <sub>16</sub> H <sub>30</sub> O <sub>2</sub>	254.2253	254.2246	0.70
56	6,10-Dimethyl-2-undecanone	C <sub>13</sub> H <sub>26</sub> O	198.1995	198.1984	1.10
60	(5 <i>E</i> )-6,10-Dimethyl-5,9-undecadien-2-one	C <sub>13</sub> H <sub>22</sub> O	194.1517	194.1518	-0.10
91	2,6,10,14-Tetramethylhexadecane	C <sub>20</sub> H <sub>42</sub>	282.3266	282.3287	-2.10
93	6,10,14-Trimethyl-2-pentadecanone	C <sub>18</sub> H <sub>36</sub> O	268.2767	268.2766	0.10
122	Squalene	C <sub>30</sub> H <sub>50</sub>	410.3971	410.3913	5.80 <sup>c</sup>
5	2-Methylpyrimidine	C <sub>5</sub> H <sub>6</sub> N <sub>2</sub>	94.0527	94.0531	-0.40
11	2,5-Dimethylpyrimidine	C <sub>6</sub> H <sub>8</sub> N <sub>2</sub>	108.0672	108.0687	-1.50
29	<i>N</i> -Methyl-2-piperidinone	C <sub>6</sub> H <sub>11</sub> NO	113.0850	113.0841	0.90
9	Dimethyl sulphone	C <sub>2</sub> H <sub>6</sub> O <sub>2</sub> S	94.0104	94.0089	1.50
18	2-Pentylfuran	C <sub>9</sub> H <sub>14</sub> O	138.1054	138.1045	0.90
124	Cholest-5-en-3β-ol	C <sub>27</sub> H <sub>46</sub> O	386.3543	386.3549	-0.60
37	3-Ethyl-4-methyl-1H-pyrrole-2,5-dione	C <sub>7</sub> H <sub>9</sub> NO <sub>2</sub>	139.0640	139.0633	0.70
38	3-Ethyl-4-methylpyrrolidine-2,5-dione	C <sub>7</sub> H <sub>11</sub> NO <sub>2</sub>	141.0795	141.079	0.50
39	3-Ethenyl-4-methyl-1H-pyrrol-2,5-dione	C <sub>7</sub> H <sub>7</sub> NO <sub>2</sub>	137.0486	137.0477	0.90
7	Unidentified	C <sub>8</sub> H <sub>12</sub> O	124.0892	124.0888	0.40
12	Unidentified	C <sub>8</sub> H <sub>10</sub> O	122.0746	122.0732	1.40

<sup>a</sup>: Values equal to or less than 2 mDa are considered significant

<sup>b</sup>: Value measured for (M - 18)<sup>+</sup> for alcohol given that the molecular ions for primary alcohols are difficult to observe

<sup>c</sup>: High mDa value probably due to the low concentration of this constituent

**Table 3.3: Enantiomeric composition<sup>a</sup> of chiral constituents of the wool and the amniotic fluid determined by GC-MS using columns C and D**

	Compounds	Enantiomeric ratio ( <i>R:S</i> )
38	3-Ethyl-4-methylpyrrolidine-2,5-dione <sup>b</sup>	54:46 <sup>c</sup> (n = 1, C <sup>d</sup> )
47	3-Methyl-2-undecanone	91:9 to 100:0 (n = 4, C)
48	Nonan-4-olide	69:31 to 78:22 (n = 5, C)
61	Decan-4-olide	58:42 to 74:26 (n = 3, C)
76	Undecan-4-olide	66:34 (n = 1, C)
109	Hexadecan-4-olide	54:46 (n = 1, D <sup>e</sup> )

<sup>a</sup> The ratios were determined by integration of single ion plots of the base peaks of the individual enantiomers. The quantitative data are given in the order of elution of the enantiomers from column C or D. In some samples, one or both of the enantiomers co-eluted with other constituents and could thus not be quantified, which accounts for the different numbers of the samples (n) shown in the table.

<sup>b</sup> Order of elution unknown.

<sup>c</sup> See discussion on p. 97

<sup>d</sup> Enantioselective column C

<sup>e</sup> Enantioselective column D

## 3.2 Characterisation of the Wool-Associated and the Amniotic Fluid Proteins

### 3.2.1 Introduction

The primary goal of the study was the identification of the volatile constituents present in the amniotic fluid of Döhne Merino ewes and the cranial wool of their lambs. This included an exploratory investigation into the possibility that proteins could also be involved in the semiochemical communication between ewe and lamb. It is known that pheromone-binding proteins (PBPs) assist in the transport of volatile pheromones in insects (Leal, 2005) and in mammals (Beynon and Hurst, 2004). The advantages of using PBPs may include a slow release mechanism by which volatile semiochemicals are released into the environment (Hurst and Beynon, 2004), the protection of labile compounds, and possibly the delivery of pheromones to specific receptor sites (Novotny *et al.*, 1999). Enzymes targeting specific molecules might also play a role in the formation of volatiles used for semiochemical communication.

The first objective of the investigation of the wool-associated proteins and the proteins present in the amniotic fluid was to establish whether these proteins have previously

been identified in mammalian secretions or excretions, and whether they are known for their ligand binding properties. The second objective was to separate the proteins without denaturing them, so as to keep the ligand-protein interaction intact, should such an interaction exist. The third objective was then to extract each separated protein and to identify the ligand(s) specifically associated with that protein.

### 3.2.2 Sample collection and preparation

Amniotic fluid and wool samples were collected as described in § 2.2 (Plates 2.1a to 2.1c). The protein content of the amniotic fluid and the wool extracts was *ca.* 0.52 µg/µl and *ca.* 0.78 µg/µl, respectively (§ 2.6). Typically, 200 µg of either the amniotic fluid proteins or the wool-associated proteins were separated on SDS-PAGE gels. Protein bands were visualised with Coomassie Brilliant Blue. Figs. 3.92 and 3.93 show typical SDS-PAGE gels for amniotic fluid proteins and wool-associated proteins.

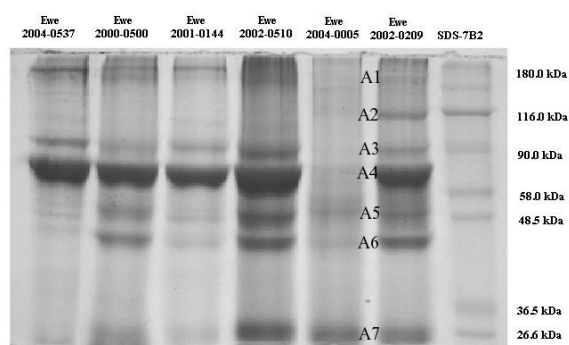


Fig. 3.92. Amniotic fluid proteins separated on 8% SDS-PAGE gel (200 µg).

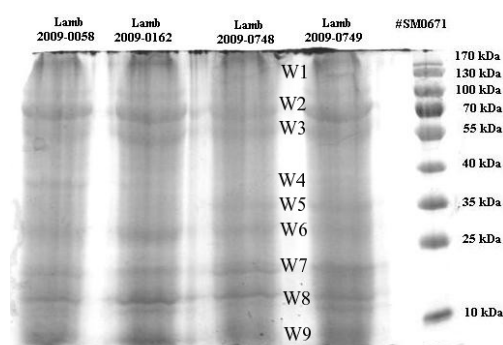


Fig. 3.93. Wool-associated proteins separated on 8% SDS-PAGE gel (200 µg).

The protein bands numbered in Figs. 3.92 and 3.93 were excised from the SDS-PAGE gel and divided into smaller fragments, and in-gel digestion with trypsin was performed according to published methods (Shevchenko *et al.*, 1996). The peptides were analysed by electrospray ionization quadrupole time-of-flight mass spectrometer (ESI-Q-TOF-MS) and the resulting data were analysed using Matrix software (<http://www.matrixscience.com>). Fragments were sequence-identified by database searches against publicly available mammalian databases using the Mascot search engine (Perkins *et al.*, 1999) and confirmed

---

---

using the Basic Local Alignment Search Tool (BLAST; Altschul *et al.*, 1990). Probability-based MOWSE (Perkins *et al.*, 1999) scores were estimated by comparing searched results against an estimated random match population, and reported as  $-10 \times \log_{10}(P)$ , with P as the absolute probability.

Amniotic fluid was subjected to anion-exchange chromatography to fractionate the proteins without denaturation. The collected protein fractions were extracted with a small volume of TBME. The organic phase was concentrated as described in § 2.3.3.1 and subjected to GC-MS analysis for ligand identification. The remaining protein-containing fractions were concentrated and the precipitated proteins of each fraction were separated on SDS-PAGE gels.

### 3.2.3 Determination of the composition of the protein fraction of the amniotic fluid

The amniotic fluid samples contained proteins with molecular weights ranging from 11 to 250 kDa. Five of the seven electrophoretically separable protein bands that were selected for peptide analysis were identified. The results are summarised in Table 3.4, and the sequences of two of the five proteins with the matched peptides are shown in Figs. 3.94 and 3.95.

Seven peptides representing 4% coverage of the bovine fibronectin (Skorstengaard, *et al.*, 1986) were identified from protein band A1 (Fig. 3.92). Fibronectin is a plasma molecule that consists of two chains connected by disulphide bonds near their carboxyl ends. Fibronectins bind cell surfaces and a number of different compounds, including collagen, fibrin, heparin, DNA and actin. These molecules are involved in cell adhesion, cell motility, opsonisation, wound healing and maintenance of cell shape (Pankov and Yamada, 2002).

Protein band A2 was identified by one peptide representing 1% coverage of glycogen phosphorylase (liver form), previously identified in mice (*Mus musculus*) (Villen *et al.*, 2007). This protein belongs to a family of oligosaccharide phosphorylases. These enzymes

catalyse the breakdown of oligosaccharides into glucose-1-phosphate units and they play an important role in carbohydrate metabolism (Voet and Voet, 1995: 484–489).

Five peptides representing 9% coverage of the serum albumin precursor of sheep (Brown *et al.*, 1989) were identified from protein bands A3, A4 and A5. A high degree of sequence conservation for albumin exists in sheep. Serum albumin is the most abundant plasma protein in mammals and it has a good binding capacity for water, Ca<sup>2+</sup>, Na<sup>+</sup>, K<sup>+</sup>, fatty acids, hormones, bilirubin and drugs. The main physiological function of serum albumin is the regulation of the colloidal osmotic pressure of blood (Metzler, 2001: 58).

```

MKWVTFISLLLLFSSAYSRGVFRDTHKSEIAHRFNDLGEENFQGLVLIAFSQYLQOCP
FDEHVKLVKELTEFAKTCVADESHAGCDKSLHTLFGDELCKVATLRETYGDMADCCEKQ
EPERNECFLNHKDDSPDLPKLKPEPDTLCAEFKADEKKFWGKYLVEVARRHPYFYAPEL
LYYANKYNGVFOECCQAEDKGACLLPKIDAMREKVLASSARQRLRCASIQKFGERALKA
WSVARLSQKFPKADFTDVTKIIVTDLTKVHKECCHGDLLECADDRADLAKYICDHQDALS
SKLKECCDKPVLEKSHCIAEVDKDAVPENLPPLTADFAEDKEVCKNYQEAKDVFLGSFL
YEYSRRHPYAVSVLLRLAKEYEATLEDCCAKEDPHACYATVFDKLKHLVDEPQNLIKK
NCELFEKHGEYGFQNALIVRYTRKAPQVSTPTLVEISRSLGKVGTKCCAKPESERMPCT
EDYLSLILNRLCVLHEKTPVSEKVTKCCTESLVNRRPCFSDLTLDETYVPKPFDEKFFT
FHADICTLPDTEKQIKKQTALVELLKHKPKATDEQLKTMENFVAFVDKCCAADDKEGC
FVLEGPKLVASTQAALA

```

Fig. 3.94. Amino acid sequence of serum albumin (*Ovis aries*). Peptides identified in the amniotic fluid are indicated in **bold letters** and the areas of high conservancy are underlined.

One peptide representing 3% coverage of the fetuin precursor of sheep (Brown *et al.*, 1992) was identified from protein band A6 (Fig. 3.92). Fetuin belongs to the  $\alpha$ -globulin family of plasma proteins. A major function of plasma proteins is to mediate the transport of a wide variety of substances in the bloodstream. Fetuin and serum albumin belong to the same group of carrier proteins (Brown *et al.*, 1992; Metzler, 2001: 58–59).

Two peptides representing 32% coverage of the immunoglobulin lambda light-chain (constant region) of sheep (Fig. 3.95) (Jenne *et al.*, 2006) were identified in protein band A7. This small polypeptide subunit is a constituent of immunoglobulins, T cell receptors, CD1 cell surface glycoproteins, secretory glycoproteins and major histocompatibility complex

(MHC) class I/II molecules, and has previously been identified in the urine of rodents (Hurst *et al.*, 2005).

```
GQPKSAPSVTLFPPSTEELSTNKATVVCLINDFYPGSVNVVWKADGSTINQNVKTTQASK  
QSNSKYAASSYLTLTGSEWKSKSSYTCEVTHEGSTVTKTVKPSECS
```

Fig. 3.95. Amino acid sequence of immunoglobulin lambda light-chain (constant region) (*Ovis aries*). Peptides identified in the amniotic fluid are indicated in **bold letters**.

### 3.2.3.1 Strong anion-exchange chromatography and ligand identification

The proteins from fresh amniotic fluid samples were fractionated by anion-exchange chromatography. The collected fractions were pooled and numbered 1 to 3, as indicated in Fig. 3.96. The elution profile consisted of one broad peak (fraction 1) of unbound protein and two partly co-eluting peaks, one sharp peak (fraction 2), and one broad peak (fraction 3). Fraction 1 did not associate with the column and did not contain any detectable proteins. The collected protein-containing fractions 2 and 3 are indicated on the chromatogram shown in Fig. 3.96.

Fig. 3.97 shows a SDS-PAGE gel of the protein-containing fractions 2 and 3. The gel in Fig. 3.97 was visually compared with the gel obtained from the protein-fractions separated by anion-exchange chromatography (Fig. 3.96) in order to tentatively identify the proteins by size estimation on SDS-PAGE, using molecular weight markers to determine the proteins' sizes. The proteins present in fraction 2 were A<sub>2</sub>2 and A<sub>2</sub>4, which corresponded to the previously identified protein glycogen phosphorylase (A<sub>2</sub>, Fig. 3.92), and the previously identified serum albumin (A<sub>4</sub>, Fig. 3.92). The proteins present in fraction 3 were present in much higher concentrations and in higher purity, and were separated into sharp bands. These proteins, A<sub>2</sub>4 and A<sub>2</sub>7, corresponded to the previously identified serum albumin (A<sub>4</sub>, Fig. 3.92) and the previously identified peptide from the immunoglobulin lambda light-chain (constant region) (A<sub>7</sub>, Fig. 3.92).



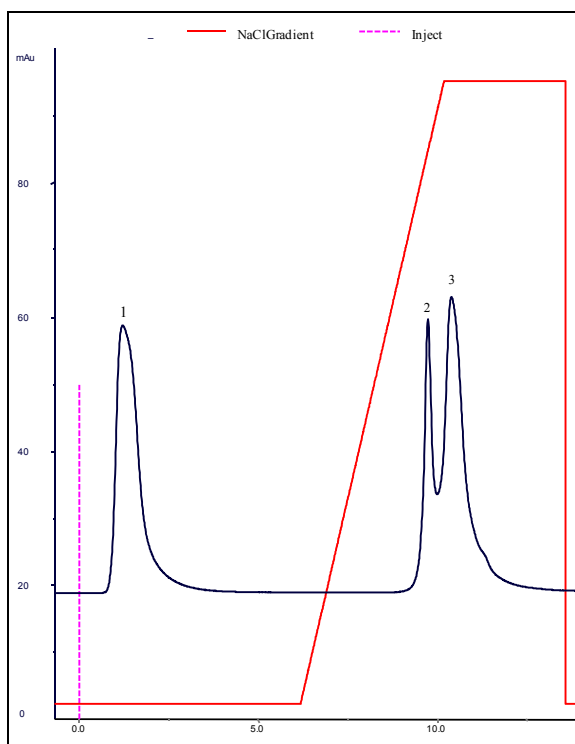


Fig. 3.96. Separation of amniotic fluid proteins by anion-exchange chromatography using a HiTrap Q column and a NaCl gradient.

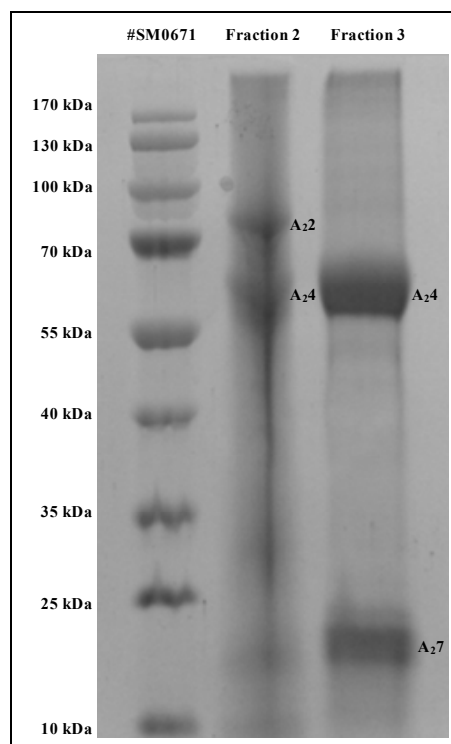


Fig. 3.97. Amniotic fluid proteins separated on 10% SDS-PAGE gel (200  $\mu$ g).

Fractions 1, 2 and 3 were extracted with TBME and the extracts concentrated and subjected to GC-MS analyses to identify any putative ligands associated with the proteins present in the collected fractions. The TBME extract of fraction 1 and 2 did not contain any extractable constituents. However, five constituents previously identified in the amniotic fluid were identified in the TIC of the TBME extract of fraction 3: hexadecanoic acid (**C104**), 9-octadecenoic acid (**C111**), octadecanoic acid (**C112**), the unidentified steroid **C123** and cholest-5-en-3 $\beta$ -ol (**C124**). No low molecular mass volatile ligands were identified in these protein-containing fractions by GC-MS analyses.

The binding specificity of serum albumin is widely known and probably accounts for the presence of the four long-chain carboxylic acids identified in fraction 3 (Bhattacharya *et al.*, 2000; Spector *et al.*, 1969). The binding capacity of serum albumin for cholest-5-en-3 $\beta$ -ol has also been demonstrated in humans (Peng *et al.*, 2008) and in rats (Deliconstantinos *et al.*, 1986) and this probably explains the presence of cholest-5-en-3 $\beta$ -ol in fraction 3.

### 3.2.4 Determination of the compositions of the wool-associated proteins

The 2.5 g wool sample contained proteins with molecular weights ranging from 70 to 11 kDa. Nine electrophoretically separable protein bands were selected for peptide analysis, from which three proteins were identified (Table 3.4). The amino acid sequences of the three selected proteins with matched peptides are shown in Figs. 3.98 to 3.100.

Eight peptides representing 16% coverage of the serum albumin precursor of sheep (Brown *et al.*, 1989) were identified in protein band W1 of the wool of Döhne Merino lambs. This protein was also identified in the amniotic fluid.

```

MKWVTFISLLLLFSSAYSRGVFRDRDTHKSEIAHRFNDLGEENFQGLVLIAFSQYLQOCP
FDEHVKLVKELTEFAKTCVADESHAGCDKSLHTLFGDELCKVATLRETYGDMADCCEKQ
EPERNECFLNHKDDSPDLPLKPKPEPDTLCAEFKADEKFFWGKLYLEVARRRHPYFYAPEL
LYYANKYNGVFOECCQAEADKGACLLPKIDAMREKVLASSARQRLRCASIQKFGERALKA
WSVARLSQKFPKADFTDVTKIIVTDLTKVHKECCHGDLLECADDRADLAKYICDHQDALS
SKLKECCDKPVLEKSHCIAEVDKDAVPENLPPLTADFAEDKEVCKNYQEAKDVFLGSFL
YEYSRRHPEYAVSVLLRLAKYEATLEDCCAKEDPHACYATVFDKLLKHLVDEPQNLIKK
NCELFEKHGGEYGFQNALIVRYTRKAPQVSTPTLVEISRSLGKVGTKCCAKPESERPCT
EDYLSLILNRLCVLHEKTPVSEKVTKCCTESLVNRRPCFSDTLTDETYVPKPFDEKFFT
FHADICTLPDTEKQIKKQTALVELLKHKPKATDEQLKTMENFVAFVDKCCAADDKEGC
FVLEGPKLVASTQAAALA

```

Fig. 3.98. Amino acid sequence of serum albumin (*Ovis aries*). Peptides identified from the wool samples are indicated in **bold letters** and the areas of high conservancy are underlined.

Five peptides representing 50% coverage of the foetal haemoglobin beta chain of sheep (Kretschmer, *et al.*, 1981) were identified in the wool of Döhne merino lambs. Globins are haem proteins that bind and transport oxygen (Voet and Voet, 1995: 216).

```

MLTAEKASVISLFAKVNVEEVGGEALGRLLVVYPWTQRFFEHFGDLSSADAILGNPKV
KGGKVLNSFSEGLKQLDDLKGAFASLSELHCDKLHVDPENFRLLGNVLVVVLARRFG
GEFTPELQANFQKVVTGVANALAHRYH

```

Fig. 3.99. Amino acid sequence of a foetal haemoglobin beta chain (*Ovis aries*). Peptides identified from the wool samples are indicated in **bold letters**.

Two peptides representing 32% coverage of the immunoglobulin lambda chain (C region) of sheep (Foley and Beh, 1989) were identified in the wool of Döhne Merino lambs. This protein was also identified in the amniotic fluid.

QPK <b>SAPSVTLFPFSKEELDTNK</b> ATVVCLISDFYPGSVNVVWKADGSIINQNVKTTQASK QSN <b>SKYAASSYLTLTGSEWK</b> SKSSYTCEVTHEGSTVTKTVKPSECS
---

Fig. 3.100. Amino acid sequence of the immunoglobulin lambda chain (C region) (*Ovis aries*). Peptides identified from the wool samples are indicated in **bold letters**.

In the case of the amniotic fluid, an investigation was carried out into ligands possibly associated with the individual proteins. In the case of the wool-associated proteins, however, only the identity of the proteins could be determined, because the method that was used to extract the proteins from the wool samples resulted in denaturation of the proteins and a concomitant loss of any possible ligands. Thus, the wool-associated proteins and the proteins present in the amniotic fluid were identified, but only the amniotic fluid proteins were further investigated for their ligand-binding properties.

### 3.2.5 Conclusions

Five proteins, fibronectin, glycogen phosphorylase (liver form), serum albumin precursor, fetuin precursor and immunoglobulin lambda chain (C region) were identified in the amniotic fluid samples collected from the bodies of newborn lambs. Three proteins, the serum albumin precursor, foetal haemoglobin (beta chain) and immunoglobulin lambda chain (C region), were identified as wool-associated proteins. Two proteins, serum albumin and an immunoglobulin protein, were identified in both the amniotic fluid and the wool. Several of the identified proteins in the amniotic fluid and those associated with the wool are known to be present in blood plasma. These could either originate from the amniotic fluid or the blood present in the amniotic fluid after the birth of the lamb, because the amniotic fluid samples were collected from the bodies of the new-born lambs. The wool samples could thus still have contained residues of the blood, amniotic fluid and saliva of the ewe. The major blood plasma protein is serum albumin, with haemoglobins, immunoglobins and fetuin also associated with blood plasma (Metzler, 2001: 58).

Several of these proteins might play a role in semiochemical communication in sheep. Fetuin and serum albumin are well characterised binding proteins; serum albumin, for example, displays good binding capacity for long-chain carboxylic acids and steroids (among other compounds). The identified lambda chain immunoglobulins are subunits of, among other compounds, MHCs. The role of MHCs in the production of pheromones used in kin recognition is well documented in the literature by, for example, Penn and Potts (1998) and Yamazaki *et al.* (1986) who demonstrated how mice can discriminate between pheromones that differ in their MHC locus. This type of discrimination has also been shown in rats (Beauchamp and Yamazaki, 2003) and in humans (Wedekind *et al.*, 1995). It is, however, not yet known whether mammals use MHCs as a marker for general kinship (Thom and Hurst, 2004) or whether MHC odours actually play a role in individual recognition (Hurst *et al.*, 2005).

In addition to the ligands hexadecanoic acid, (*Z*)-9-octadecenoic acid, octadecanoic acid and cholest-5-en-3 $\beta$ -ol, previously identified as ligands of serum albumin (Deliconstantinos *et al.*, 1986; Saifer and Goldman, 1961;) the unidentified steroid **C123** was also found as a ligand of serum albumin in this study. The paucity of ligands of higher volatility in the material analysed in the present study could be attributed to the low concentrations in which the volatile compounds are present in the amniotic fluid. In future research the identified proteins could be cloned and expressed in a bacterial or yeast system and purified to assess their ligand-binding abilities by exposing them to the volatile constituents identified in this study.

To determine the pheromone-binding properties of the wool-associated proteins, it could be attempted to devise a method that would make it possible to remove the wool-associated proteins without the denaturation and concomitant loss of any putative ligands.

**Table 3.4: Proteins in the amniotic fluid of Döhne Merino ewes and in the wool of their lambs**

Band no.	MASCOT/BLAST result	Accession number	Theoretical molecular mass (kDa)	MASCOT MOWSE score <sup>a</sup>	NCBI BLAST bit score <sup>b</sup>	NCBI BLAST E-value <sup>c</sup>	Percentage coverage <sup>d</sup>
Amniotic fluid proteins							
A1	Fibronectin ( <i>Bos taurus</i> )	NP_001157250	249.401	237	49.3	1e <sup>-04</sup>	4 (7)
A2	Glycogen phosphorylase, liver form ( <i>Mus musculus</i> )	NP_573461	97.238	54	46.0	8e <sup>-06</sup>	1 (1)
A3, A4, A5	Serum albumin precursor ( <i>Ovis aries</i> )	NP_001009376	69.143	355	51.6	6e <sup>-09</sup>	9 (5)
A6	Fetuin precursor ( <i>Ovis aries</i> )	NP_001009802	38.655	78	45.2	2e <sup>-07</sup>	3 (1)
A7	Immunoglobulin lambda light chain constant region segment 1 ( <i>Ovis aries</i> )	AAU45093	11.322	166	43.1	2e <sup>-06</sup>	32 (2)
Wool-associated proteins							
W1, W2	Serum albumin precursor ( <i>Ovis aries</i> )	NP_001009376	69.143	394	64.3	1e <sup>-12</sup>	16 (8)
W5, W6	Haemoglobin beta chain, foetal ( <i>Ovis aries</i> )	P02083	15.921	329	63.5	1e <sup>-12</sup>	50 (5)
W9	Immunoglobulin lambda chain C region ( <i>Ovis aries</i> )	B30554	11.305	169	45.4	4e <sup>-07</sup>	32 (2)

<sup>a</sup> Probability-based MOWSE score: Ions score is  $-10 \times \log(P)$ , where P is the probability that the observed match is a random event. Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits. Individual ions scores indicating identity or extensive homology ( $p < 0.05$ ) for each of the proteins were as follows: A1  $\geq 38$ ; A2  $\geq 39$ ; A3, A4, A5, and A6  $\geq 37$ ; A7  $\geq 38$ ; W1, W2, W5, W6 and W9  $\geq 37$ .

<sup>b</sup> Bit score rates the alignment of the peptide(s) with the protein sequence. High bit scores indicate good alignments. Bit scores are calculated to take into account the alignment of similar or identical residues and any gaps introduced to align the sequence.

<sup>c</sup> E-value indicates the statistical significance of the peptide and protein alignment, and reflects the size of the database and the scoring system used. The lower the E-value the more significant the hit. A sequence alignment with an E-value of 0.10 indicates that the similarity has a 10 in 100 probability of occurring by chance.

<sup>d</sup> Number of peptides identified is indicated in brackets.



a. Ewe licking the amniotic fluid off her newborn lamb.



b. Ewe eating the foetal membrane and amniotic fluid off her newborn lamb.



c. Ewe sniffing at the nose and mouth of a lamb.



d. Ewe sniffing at the neck of an alien lamb.



e. Ewe sniffing at the nose of an alien lamb.



f. Ewe sniffing at the body of a lamb.

## Plate 3.1





a. Ewe sniffing at a lamb's hind quarters.



b. Ewe sniffing at an alien lamb's neck.



c. Ewe sniffing at an alien lamb's hind quarters.



d. Ewe sniffing at the neck of an alien lamb.



e. Ewe sniffing at an alien lamb's head.

## Plate 3.2

---

---

## References

- Alexander, G., “Odour, and the recognition of lambs by Merino ewes”, *Appl. Anim. Ethol.* **4**: 153–158 (1978).
- Alexander, G. and Shillito, E. E., “The importance of odour, appearance and voice in maternal recognition of the young in Merino sheep (*Ovis aries*)”, *Appl. Anim. Ethol.* **3**: 127–135 (1977).
- Alexander, G. and Stevens, D., “Recognition of washed lambs by Merino ewes”, *Appl. Anim. Ethol.* **7**: 77–86 (1981).
- Alexander, G., Stevens, D. and Bradley, L. R., “Washing lambs and confinement as aids to fostering”, *Appl. Anim. Ethol.* **10**: 251–261 (1983).
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J., “Basic local alignment search tool”, *J. Mol. Biol.* **215**: 403–410 (1990).
- Bhattacharya, A. A., Grüne, T. and Curry, S., “Crystallographic analysis reveals common modes of binding of medium and long-chain fatty acids to human serum albumin”, *J. Mol. Biol.* **303**: 721–732 (2000).
- Beauchamp, G. K. and Yamazaki, K., “Chemical signalling in mice”, *Biochem. Soc. Trans.* **31**: 147–151 (2003).
- Beynon, R. J. and Hurst, J. L., “Urinary proteins and the modulation of chemical scents in mice and rats”, *Peptides* **25**: 1553–1563 (2004).
- Brennan, P. A. and Kendrick, K. M., “Mammalian social odours: attraction and individual recognition”, *Phil. Trans. R. Soc. B* **361**: 2061–2078 (2006).



Brown, W. M., Dziegielewska, K. M., Foreman, R. C. and Saunders, N. R., "Nucleotide and deduced amino acid sequence of sheep serum albumin", *Nucleic Acids Res.* **17**: 10495 (1989)

Brown, W. M., Dziegielewska, K. M., Saunders, N. R., Christie, D. L., Nawratil, P. and Mueller-Esterl, W., "The nucleotide and deduced amino acid structures of sheep and pig fetuin. Common structural features of the mammalian fetuin family", *Eur. J. Biochem.* **205**: 321–331 (1992).

Budzikiewicz, H., Djerassi, C. and Williams, D. H., "Mass Spectrometry of Organic Compounds", Holden-Day Inc., San Francisco (1967).

Burger, B. V., "Mammalian Semiochemicals" in "The Chemistry of Pheromones and Other Semiochemicals II", Schultz, S. (ed.), Topics in Current Chemistry, Springer, Berlin, **240**: 231–278 (2005a).

Burger, B. V., "Mass Spectrometry in Pheromone Research" in "Elsevier's Encyclopedia of Mass Spectrometry", Nibbering, N. (ed.) **4**: 852–863 (2005b).

Burger, B.V., Marx, B., Le Roux, M. and Burger, W. J. G., "Simplified analysis of organic compounds in headspace and aqueous samples by high-capacity sample enrichment probe", *J. Chrom. A* **1121**: 259–267 (2006).

Burger, B. V., Viviers, M. Z., Bekker, J. P. I., Le Roux, M., Fish, N., Fourie, W. B. and Weibchen, G., "Chemical characterization of territorial marking fluid of male Bengal tiger, *Panthera tigris*", *J. Chem. Ecol.* **34**: 659–671 (2008).

Buser, H-R., Arn, H., Guerin, P. and Rauscher, S., "Determination of double bond position in mono-unsaturated acetates by mass spectrometry of dimethyl disulfide adducts", *Anal. Chem.* **55**: 818–822 (1983).

Christiansen, K., Mahadevan, V., Viswanathan, C. V. and Holman, R. T., “Mass spectrometry of long-chain aliphatic aldehydes, dimethyl acetals and alk-1-enyl ethers”, *Lipids* **4**: 421–427 (1969).

Cronje, C., “Chemiese Karakterisering van die Aroma van die Heuningbossesie *Cyclopia genistoides*”, MSc thesis, University of Stellenbosch (2006).

Deliconstantinos, G., Tsopanakis, C., Karayiannakos, P. and Skalkeas, G., “Evidence for the existence of non-esterified cholesterol carried by albumin in rat serum”, *Atherosclerosis* **61**: 67–75 (1986).

Díaz, O., Fernández, M., García de Fernando, G. D., De la Hoz, L. and Ordóñez, J. A., “Effect of the addition of Pronase E on the proteolysis in dry fermented sausages”, *Meat Sci.* **34**: 205–216 (1993).

Dulac, C. and Torello, A. T., “Molecular detection of pheromone signals in mammals: from genes to behaviour”, *Nat. Rev. Neurosci.* **4**: 551–562 (2003).

Foley, R. C. and Beh, K. J., “Isolation and sequence of sheep Ig H and L chain cDNA”, *J. Immunol.* **142**: 708–711 (1989).

Hagen, B. F., Berdagué, J-L., Holck, A. L., Naes, H. and Blom, H., “Bacterial proteinase reduces maturation time of dry fermented sausages”, *J. Food Sci.* **61**: 1024–1029 (1996).

Harrison, A. G., “The high-resolution mass spectra of aliphatic aldehydes”, *Org. Mass Spectrom.* **3**: 549–555 (1970).

Herranz, B., Fernández, M., De la Hoz, L. and Ordóñez, J. A., “Use of bacterial extracts to enhance amino acid breakdown in dry fermented sausages”, *Meat Sci.* **72**: 318–325 (2006).

Herrero, M. A., Kreamsner, J. M. and Kappe, C. O., "Nonthermal microwave effects revisited: On the importance of internal temperature monitoring and agitation in microwave chemistry", *J. Org. Chem.* **73**: 36–47 (2008).

Houpt, K. A., "Domestic Animal Behaviour for Veterinarians and Animal Scientists", Fourth Edition, Blackwell Publishing, Oxford (2005).

Hurst, J. L. and Beynon, R. J., "Scent wars: the chemobiology of competitive signalling in mice", *BioEssays* **26**: 1288–1298 (2004).

Hurst, J. L., Thom, M. D., Nevison, C. M., Humphries, R. E. and Beynon, R. J., "MHC odours are not required or sufficient for recognition of individual scent owners", *Proc. Biol. Sci.* **272**: 715–724 (2005).

Jenne, C. N., Kennedy, L. J. and Reynolds, J. D., "Antibody repertoire development in the sheep", *Dev. Comp. Immunol.* **30**: 165–174 (2006).

Kendrick, K. M., Lévy, F. and Keverne, E. B., "Changes in the sensory processing of olfactory signals induced by birth in sheep", *Science* **256**: 833–836 (1992).

Kenig, F., Simons, D-J. H., Crich, D., Cowen, J. P., Ventura, G. T. and Rehbein-Khalily, T., "Structure and distribution of branched aliphatic alkanes with quaternary carbon atoms in Cenomanian and Turonian black shales of Pasquia Hills (Saskatchewan, Canada)", *Org. Geochem.* **36**: 117–138 (2005).

Kovats, E., "Gas-chromatographische Charakterisierung organischer Verbindungen. Teil 1: Retentionsindices aliphatischer Halogenide, Alkohole, Aldehyde und Ketone", *Helv. Chim. Acta* **41**: 1915–1932 (1958).

Kretschmer, P. J., Coon, H. C., Davis, A., Harrison, M. and Nienhuis, A. W., "Hemoglobin switching in sheep. Isolation of the fetal  $\gamma$ -globin gene and

demonstration that the fetal  $\gamma$ - and adult  $\beta^A$ -globin genes lie within eight kilobase segments of homologous DNA”, *J. Biol. Chem.* **256**: 1975–1982 (1981).

Le Roux, M., “Reuksintuiglike Kommunikasie: Chemiese Samestelling van Eksokriene Klierafskeidings van die Bontebok (*Damaliscus dorcas dorcas*), Springbok (*Antidorcas marsupialis*) en Grysbok (*Raphicerus melanotis*), PhD thesis, University of Stellenbosch (1980).

Leal, W. S., “Pheromone reception” in “The Chemistry of Pheromones and Other Semiochemicals”, Schultz, S. (ed.), “Topics in Current Chemistry”, **240** pp. 1–36 (2005).

Lévy, F., Keller, M. and Poindron, P., "Olfactory regulation of maternal behavior in mammals", *Horm. Behav.* **46**: 284–302 (2004).

Lisovac, A. M. and Shooter, D., “Volatiles from sheep wool and the modification of wool odours”, *Small Ruminant Res.* **49**: 115–124 (2003).

Lynch, J. J, Hinch, G. N. and Adams, D. B., “The Behaviour of Sheep. Biological Principles and Implications for Production”, C·A·B International and CSIRO Australia, Melbourne, 1992.

Maas, B., Dietrich, A. and Mosandl, A., “Collection of enantiomer separation factors obtained by capillary gas chromatography on chiral stationary phases”, *J. High Resolut. Chrom.* **17**: 109–115 (1994).

Maxwell, J. R, Douglas, A. G., Eglinton, G. and McCormick, A., “The botryococcenes–hydrocarbons of novel structure from the alga *Botryococcus braunii*, Kützing”, *Phytochemistry* **7**: 2157–2171 (1968).

---

---

McLafferty, F. W. and Tureček, F., "Interpretation of Mass Spectra", Fourth Edition, University Science Books, California (1993).

Metzler, D. E., "Biochemistry. The Chemical Reactions of Living Cells", Second Edition, Volume 1, Harcourt Academic Press, San Diego (2001).

Motiuk, K., "Wool wax acids: a review", *J. Am. Oil Chem. Soc.* **56**: 91–97 (1979a).

Motiuk, K., "Wool wax alcohols: a review", *J. Am. Oil Chem. Soc.* **56**: 651–658 (1979b).

Motiuk, K., "Wool wax hydrocarbons: a review", *J. Am. Oil Chem. Soc.* **57**: 145–146 (1980).

Narahashi, Y., "Pronase", *Methods Enzymol.* **19**: 651–664 (1970).

Novotny, M. V., Ma, W., Wiesler, D. and Zidek, L., "Positive identification of the puberty-accelerating pheromone of the house mouse: the volatile ligands associating with the major urinary protein", *Proc. Biol. Sci.* **266**: 2017–2022 (1999).

Olivier, J. J., Shepherd, Computerised flock-recording program, ARC Animal Improvement Institute, RSA (2003).

Pankov, R. and Yamada, K. M., "Fibronectin at a glance", *J. Cell Sci.* **115**: 3861–3863 (2002).

Pavia, D. L., Lampman, G. M. and Kriz, G. S., "Introduction to Spectroscopy. A guide for Students of Organic Chemistry", Third Edition, Brooks/Cole Thomson Learning, USA (2001).

---

---

Peng, L., Minbo, H., Fang, C., Xi, L. and Chaocan, Z., "The interaction between cholesterol and human serum albumin", *Protein Pept. Lett.* **15**: 360–364 (2008).

Penn, D. and Potts, W. K., "Untrained mice discriminate MHC-determined odors", *Physiol. Behav.* **64**: 235–243 (1998).

Perkins, D. N., Pappin, D. J., Creasy, D. M. and Cottrell, J. S., "Probability-based protein identification by searching sequence databases using mass spectrometry data", *Electrophoresis* **20**: 3551–3567 (1999).

Poindron, P., Gilling, G., Hernandez, H., Serafin, N. and Terrazas, A., "Early recognition of newborn goat kids by their mother: 1. Non-olfactory discrimination", *Dev. Psychobiol.* **43**: 82–89 (2003).

Poindron, P., Lévy, F. and Keller, M., "Maternal responsiveness and maternal selectivity in domestic sheep and goats: the two facets of maternal attachment", *Dev. Psychobiol.* **49**: 54–70 (2007).

Price, E. O., Dunn, G. C., Talbot, J. A. and Dally, M. R., "Fostering lambs by odor transfer: the substitution experiment", *J. Anim. Sci.* **59**: 301–307 (1984).

Rietdorf, M., "Identifizierung und Synthese flüchtiger Substanzen aus Säugetieren", PhD thesis, Institut für Organische Chemie, Hamburg (2002).

Rigaudy, J. and Klesney, S. P., "Nomenclature of Organic Chemistry. Sections A, B, C, D, E, F and H", Pergamon Press, Oxford (1979).

Saifer, A. and Goldman, L., "The free fatty acids bound to human serum albumin", *J. Lipid Res.* **2**: 268–270 (1961).

---

---

Schlossman, M. L. and McCarthy, J. P., “Lanolin and derivatives chemistry: relationship to allergic contact dermatitis”, *Contact Dermatitis* **5**: 65–72 (1979).

Sghendo, L., Mifsud, J., Ellul-Micallef, R., Portelli, J. and Millership, J. S., “A sensitive gas chromatographic / mass spectrometric method for the resolution and quantification of ethosuximide enantiomers in biological fluids”, *J. Chromatogr. B* **772**: 307–315 (2002).

Sharkey, A. G., Schultz, J. L. and Friedel, R. A., “Mass Spectra of Ketones”, *Anal. Chem.* **28**: 934–940 (1956).

Shevchenko, A., Wilm, M., Vorm, O. and Mann, M., “Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels”, *Anal. Chem.* **68**: 850–858 (1996).

Silverstein, R. M., Bassler, G. C. and Morrill, T. C., “Spectrometric Identification of Organic Compounds”, John Wiley & Sons, Inc., New York (1991).

Skorstengaard, K., Jensen, M. S., Sahl, P., Petersen, T. E. and Magnusson, S., “Complete primary structure of bovine plasma fibronectin”, *Eur. J. Biochem.* **161**: 441–453 (1986).

Smit, D., “Olfactory Communication: Chemical Characterization of the Ventral Gland and Supplementary Sacculi Secretions of the Dwarf Hamster, *Phodopus sungorus sungorus*”, PhD thesis, University of Stellenbosch (1997).

Soini, H. A., Bruce, K. E., Wiesler, D., David, F., Sandra, P. and Novotny, M. V., “Stir bar sorptive extraction: a new quantitative and comprehensive sampling technique for determination of chemical signal profiles from biological media”, *J. Chem. Ecol.* **31**: 377–392 (2005).

Spector, A. A., John, K. and Fletcher, J. E., "Binding of long-chain fatty acids to bovine serum albumin", *J. Lipid Res.* **10**: 56–67 (1969).

Spiteller, G., "Interpretation of Mass Spectra Obtained from Natural Sources Especially those of Aliphatic Compounds", Course given at the University of Stellenbosch (1994).

Thom, M. D. and Hurst, J. L., "Individual recognition by scent", *Ann. Zool. Fennici.* **41**: 765–787 (2004).

Tien, F-C., "Structure Elucidation of Constituents of the Preorbital Secretion of the Grysbok, *Raphicerus melanotis*", MSc thesis, University of Stellenbosch (1993).

Villén, J., Beausoleil, S. A., Gerber, S. A. and Gygi, S. P., "Large-scale phosphorylation analysis of mouse liver", *Proc. Natl. Acad. Sci. U.S.A.* **104**: 1488–1493 (2007).

Vincenti, M., Guglielmetti, G., Cassani, G. and Tonini, C., "Determination of double bond position in diunsaturated compounds by mass spectrometry of dimethyl disulfide derivatives", *Anal. Chem.* **59**: 694–699 (1987).

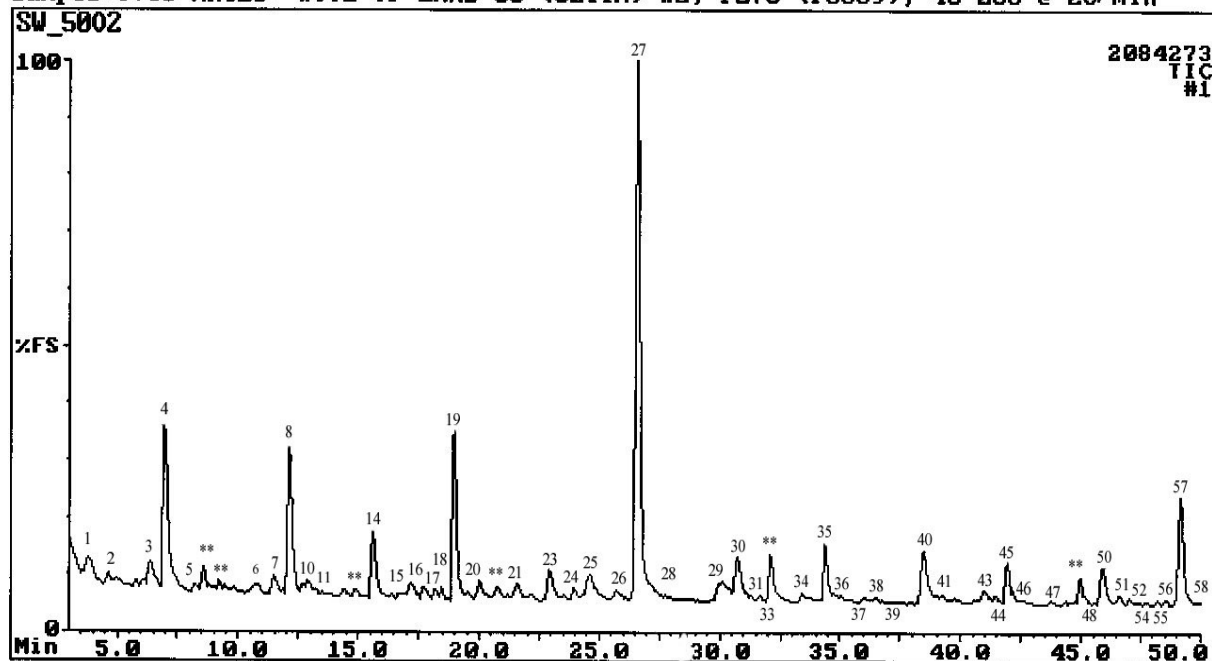
Voet, D. and Voet, J. G., "Biochemistry", Second Edition, John Wiley & Sons, Inc., New York (1995).

Wedekind, C., Seebeck, T., Bettens, F. and Paepke, A. J., "MHC-dependent mate preferences in humans", *Proc. R. Soc. London B* **260**: 245–249 (1995).

Yamazaki, K., Beauchamp, G. K., Matsuzaki, O., Kupniewski, D., Bard, J., Thomas, L. and Boyse, E. A., "Influence of a genetic difference confined to mutation of H-2K on the incidence of pregnancy block in mice", *Proc. Natl. Acad. Sci. USA* **83**: 740–741 (1986).



Sample: OVIS ARIES: WOOL OF LAMB 50 (3211A) #2, P275 (PS089), 40-280 @ 2C/min



Sample: OVIS ARIES: WOOL OF LAMB 50 (3211A) #2, P275 (PS089), 40-280 @ 2C/min

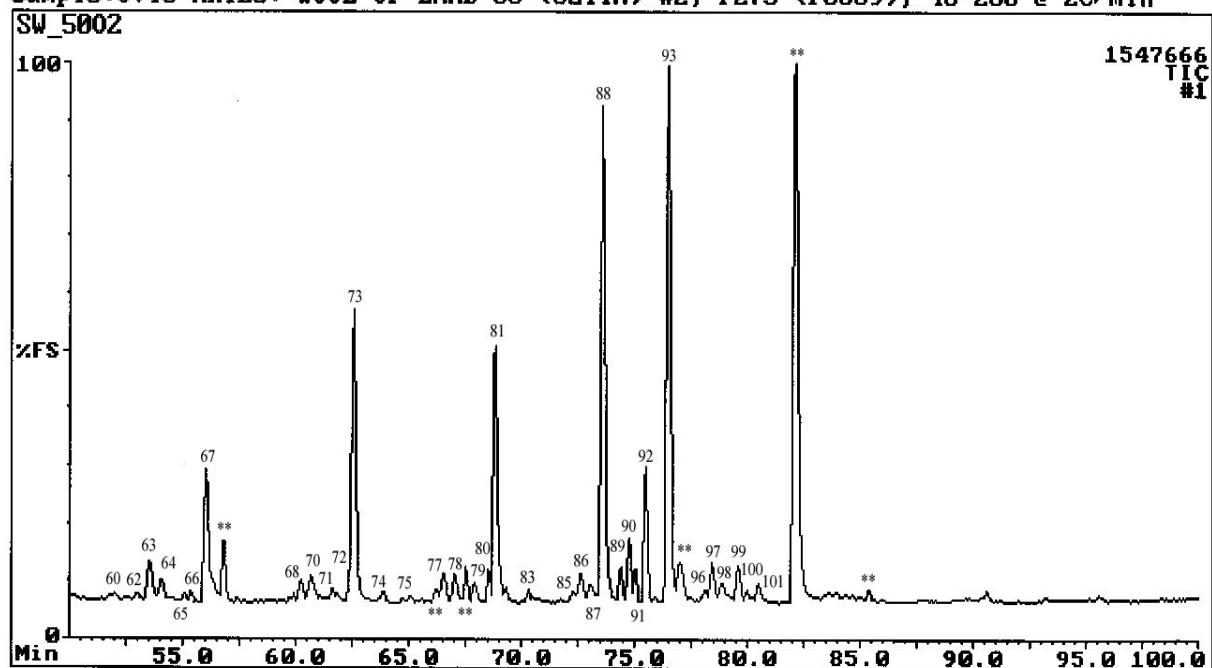


Fig. 3.12. TIC of the headspace of the wool of lamb US-2007-0050.



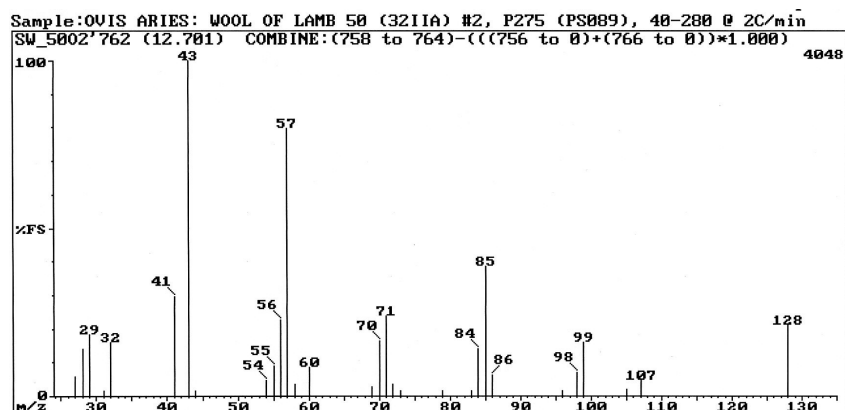


Fig. 3.14. The EI mass spectrum of nonane (C10).

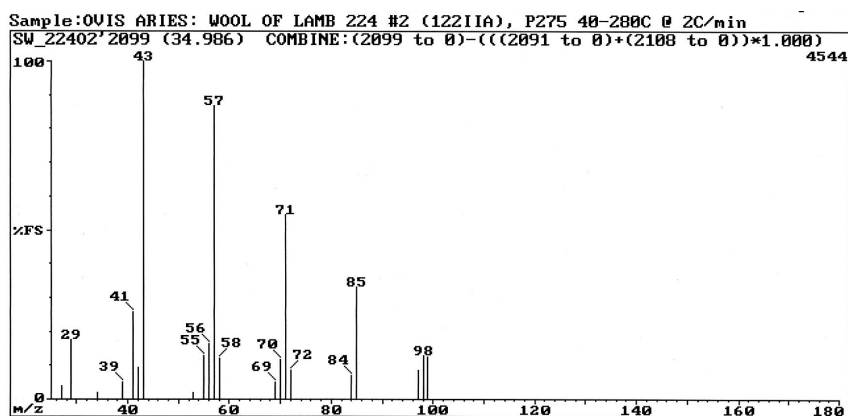


Fig. 3.15. The EI mass spectrum of dodecane (C36).

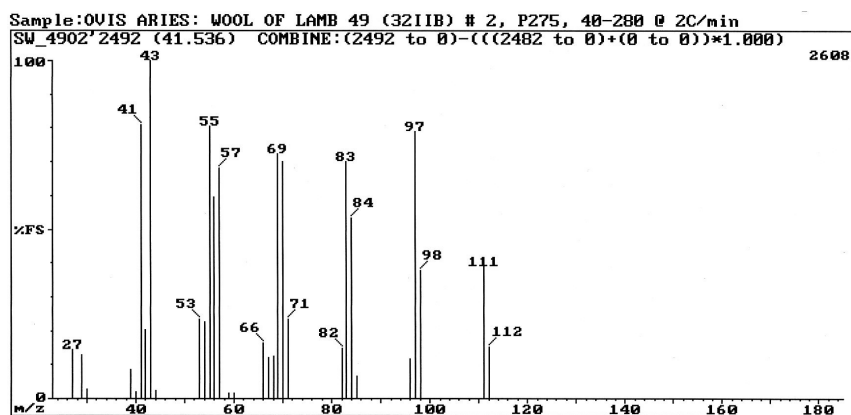


Fig. 3.16. The EI mass spectrum of 1-tridecene (C44).

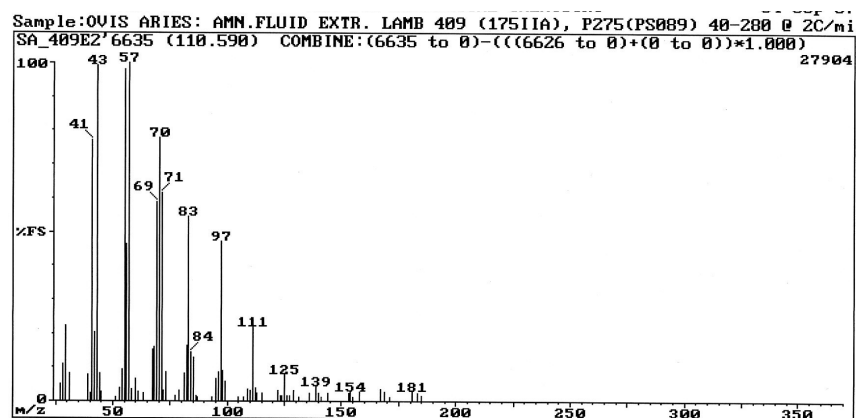


Fig. 3.17. The EI mass spectrum of 1-hexacosene (C120).

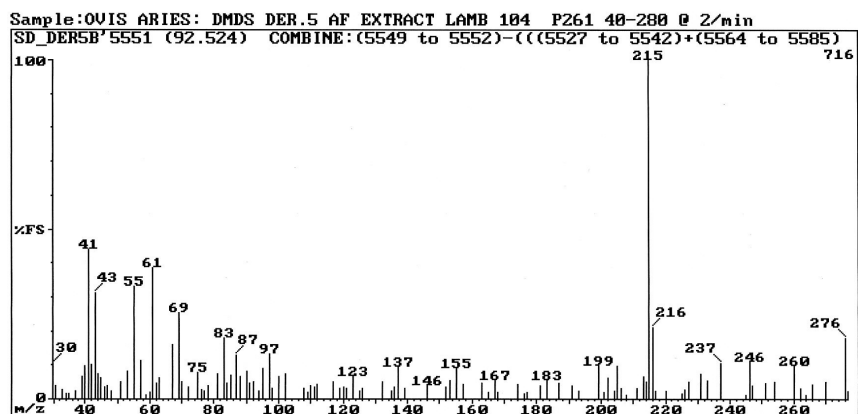


Fig. 3.18. The EI mass spectrum of DMDS adduct of 1-tridecene (C44).

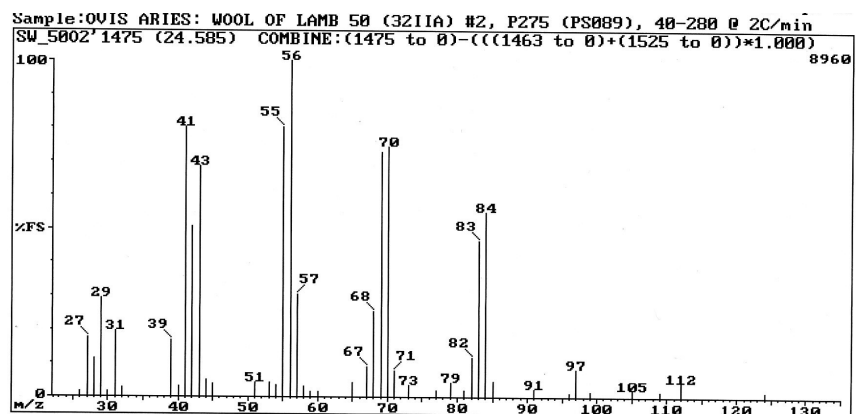


Fig. 3.19. The EI mass spectrum of 1-octanol (C25).

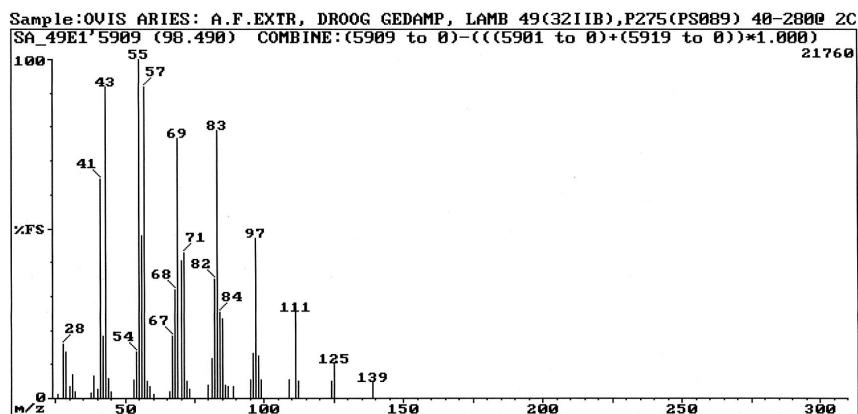


Fig. 3.20. The EI mass spectrum of 1-icosanol (C114).

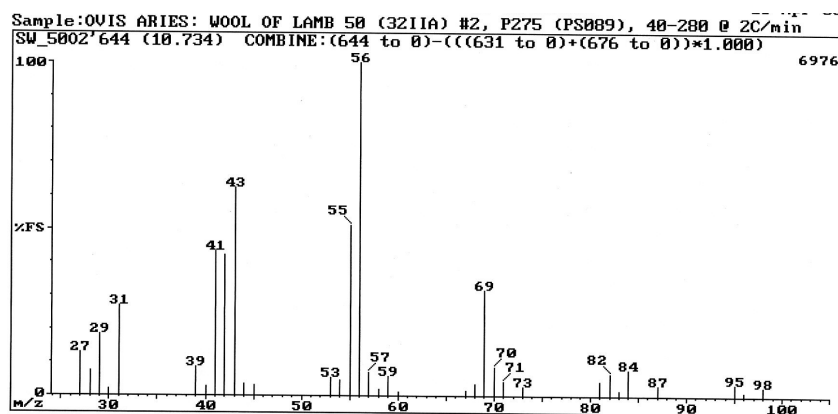


Fig. 3.21. The EI mass spectrum of 4-methyl-1-pentanol (C6).

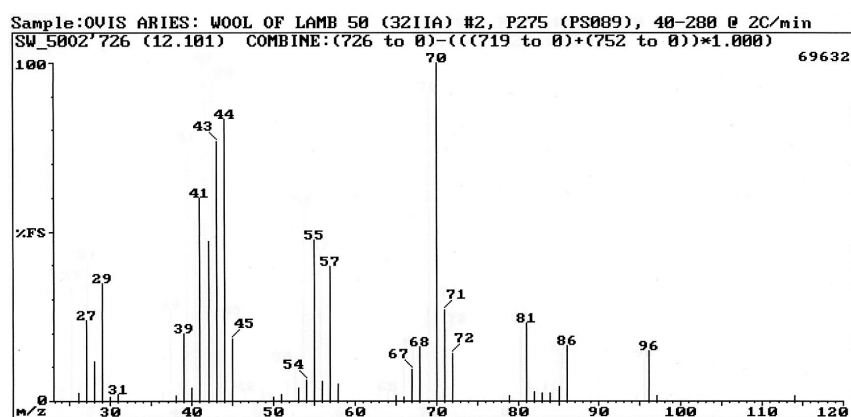


Fig. 3.22. The EI mass spectrum of heptanal (C8).

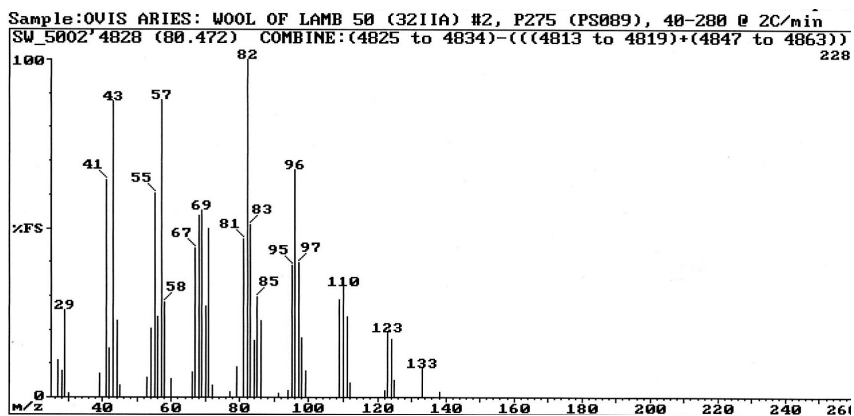


Fig. 3.23. The EI mass spectrum of heptadecanal (C101).

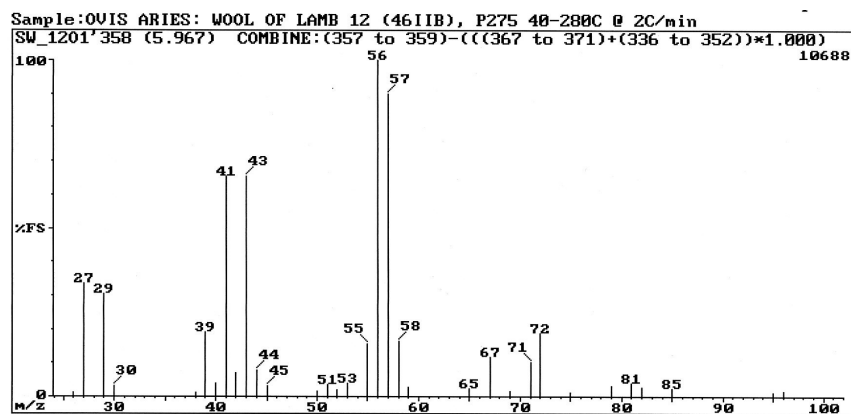


Fig. 3.24. The EI mass spectrum of 3-methylpentanal (C1).

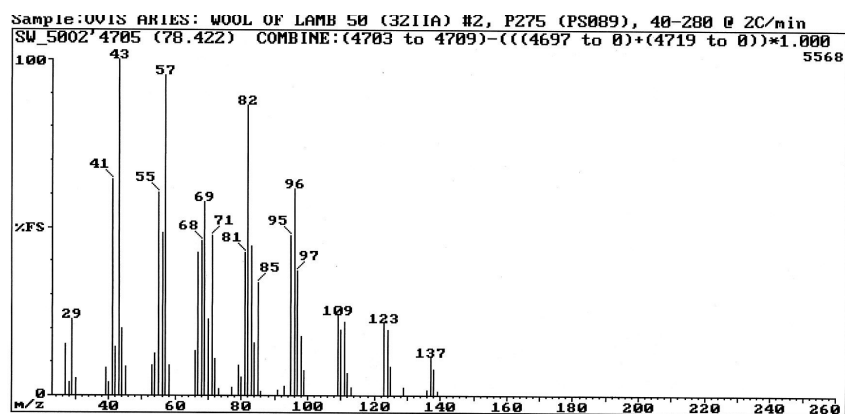


Fig. 3.25. The EI mass spectrum of 15-methylhexadecanal (C97).

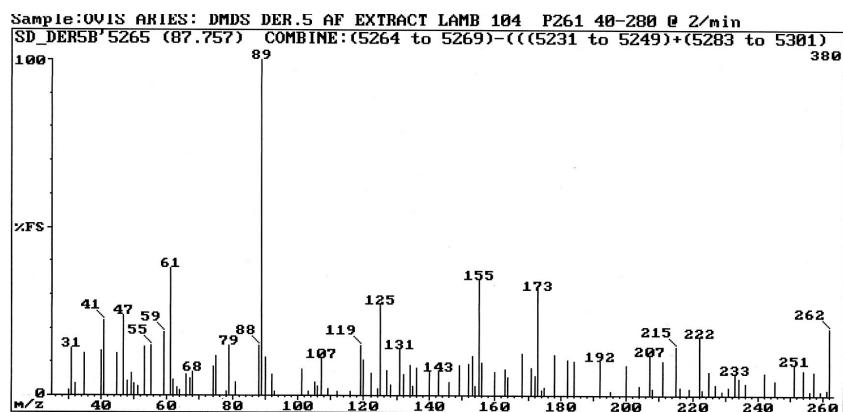
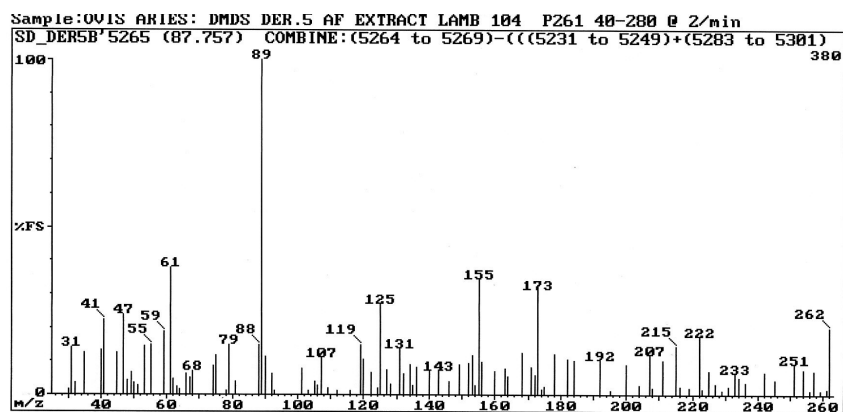
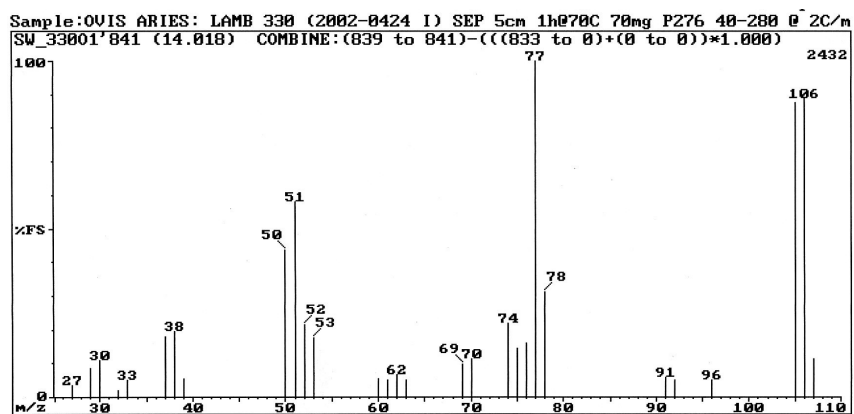
Fig. 3.26. The EI mass spectrum of (*E*)-2-undecenal (C50).Fig. 3.27. The EI mass spectrum of DMDS adduct of (*E*)-2-undecenal (C50).

Fig. 3.28. The EI mass spectrum of benzaldehyde (C13).

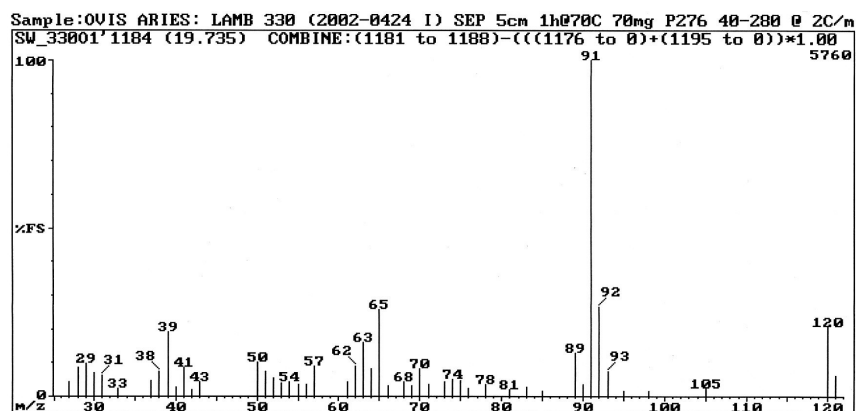


Fig. 3.29. The EI mass spectrum of phenylacetaldehyde (C22).

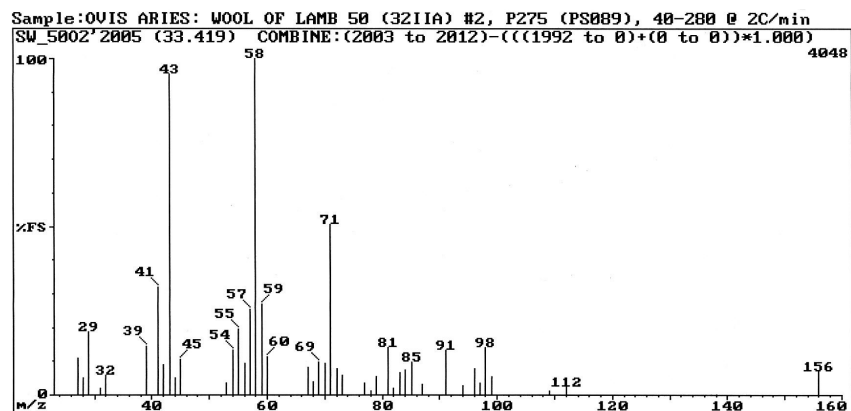


Fig. 3.30. The EI mass spectrum of 2-decanone (C34).

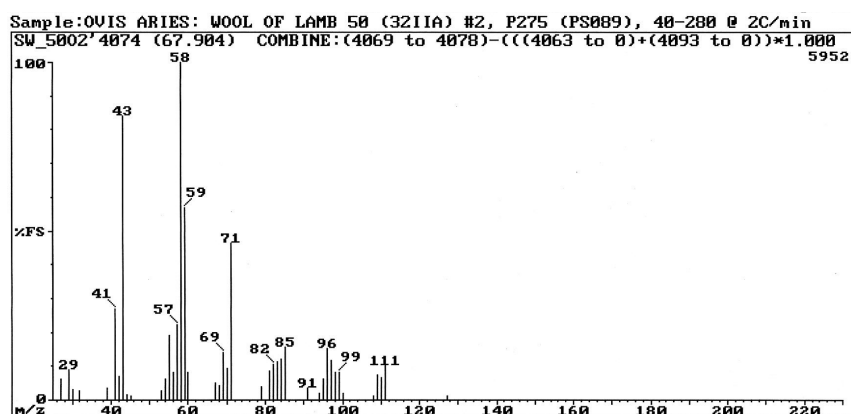


Fig. 3.31. The EI mass spectrum of 2-pentadecanone (C79).



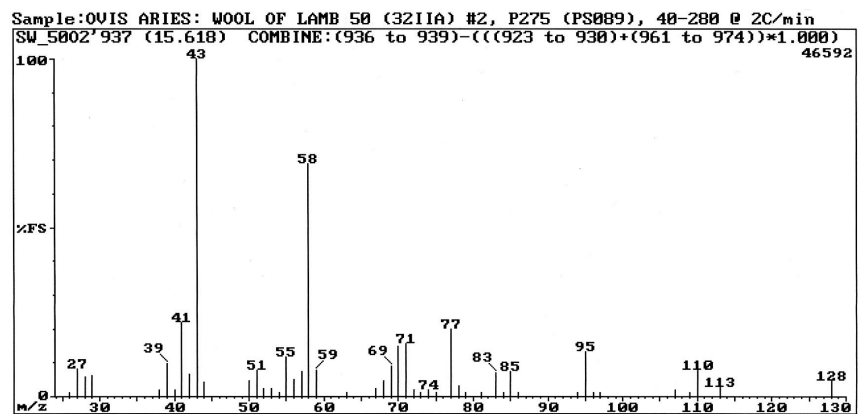


Fig. 3.32. The EI mass spectrum of 6-methyl-2-heptanone (C14).

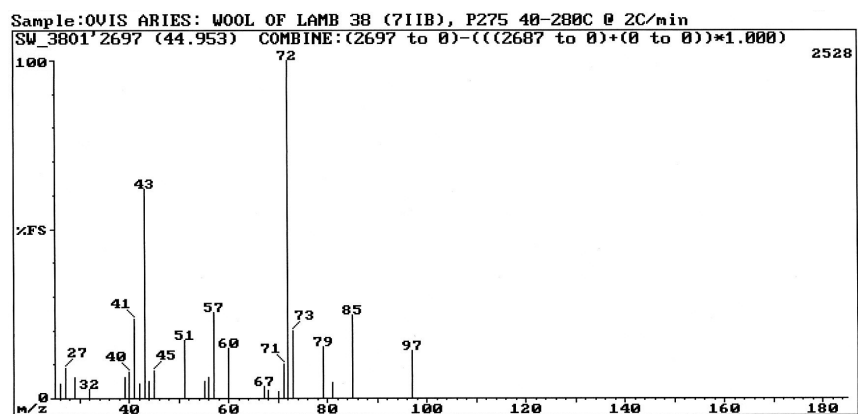


Fig. 3.33. The EI mass spectrum of 3-methyl-2-undecanone (C47).

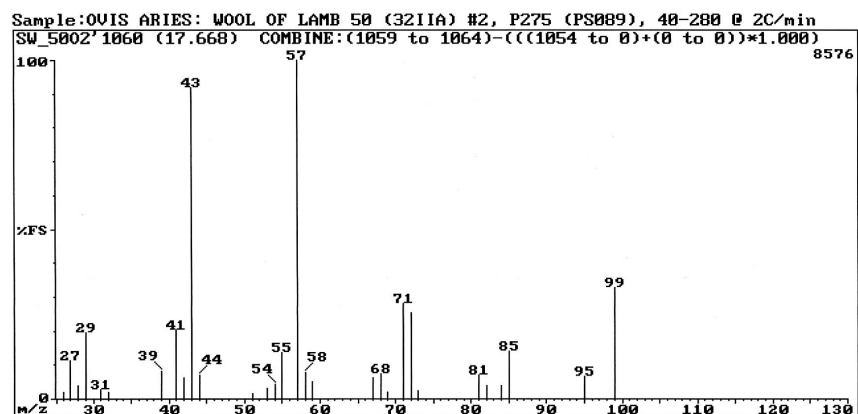


Fig. 3.34. The EI mass spectrum of 3-octanone (C16).

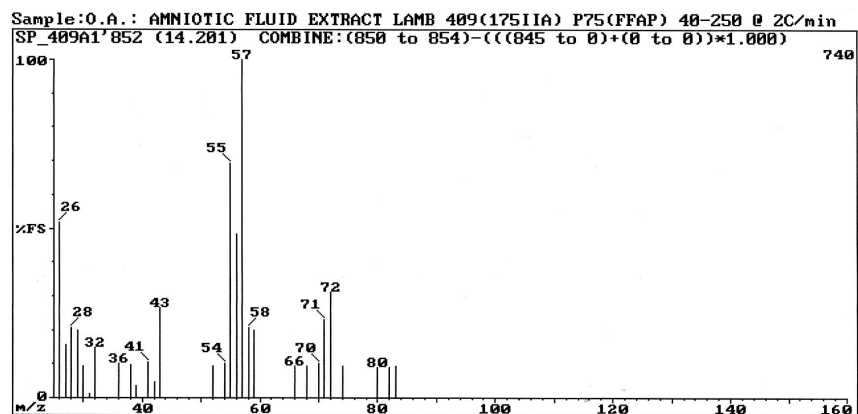


Fig. 3.35. The EI mass spectrum of 3-decanone (CP4).

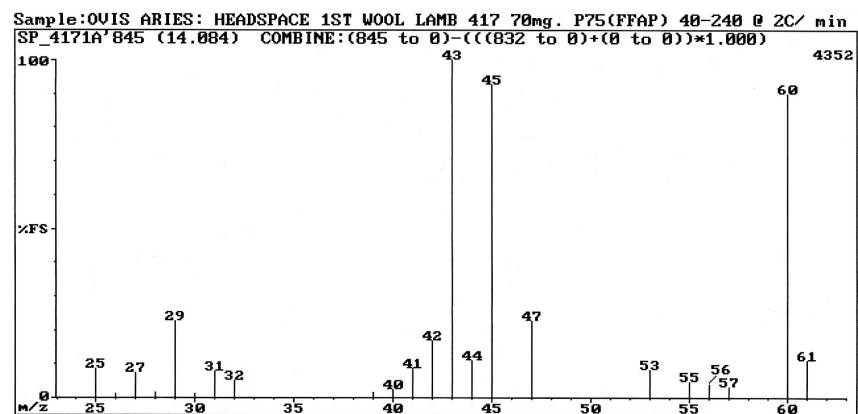


Fig. 3.36. The EI mass spectrum of acetic acid (CP3).

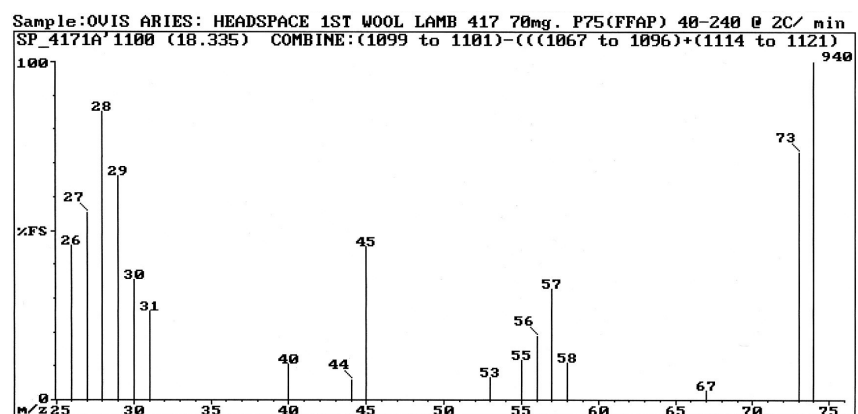


Fig. 3.37. The EI mass spectrum of propanoic acid (CP5).

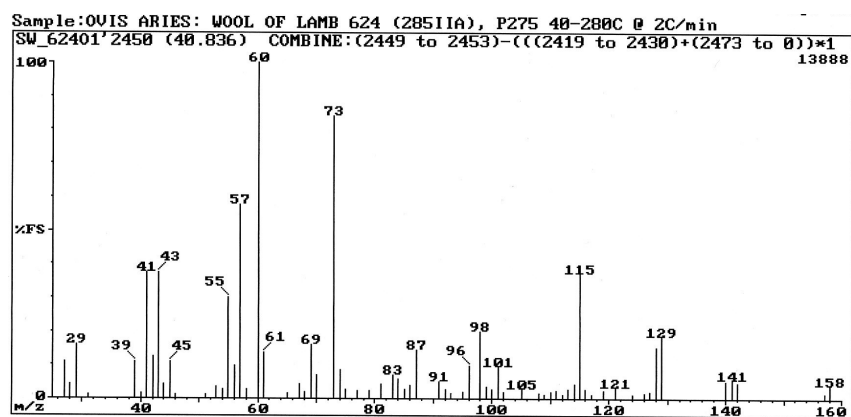


Fig. 3.38. The EI mass spectrum of nonanoic acid (C42).

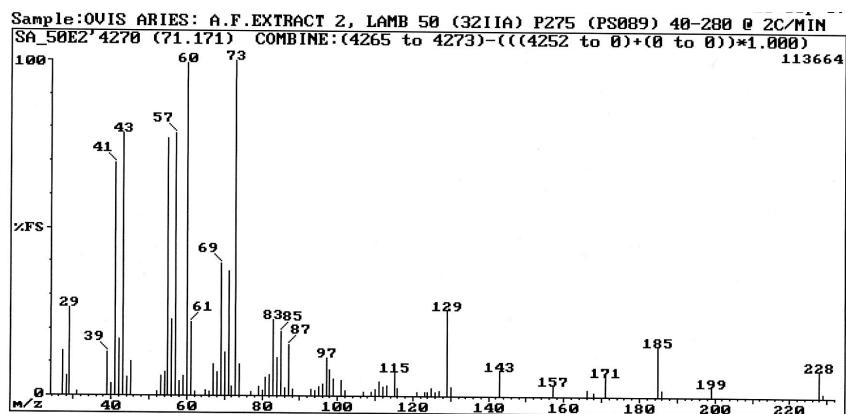


Fig. 3.39. The EI mass spectrum of tetradecanoic acid (C85).

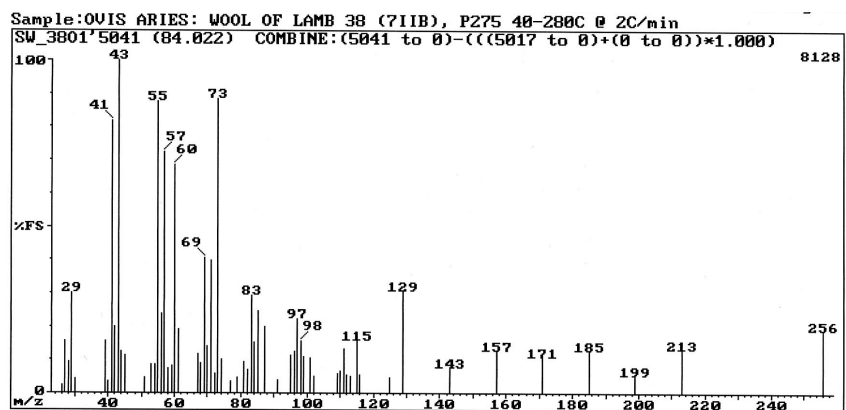


Fig. 3.40. The EI mass spectrum of hexadecanoic acid (C104).

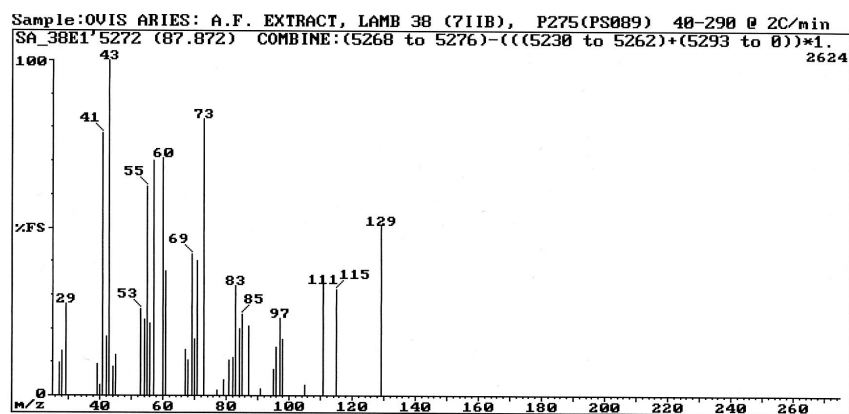


Fig. 3.41. The EI mass spectrum of heptadecanoic acid (C107).

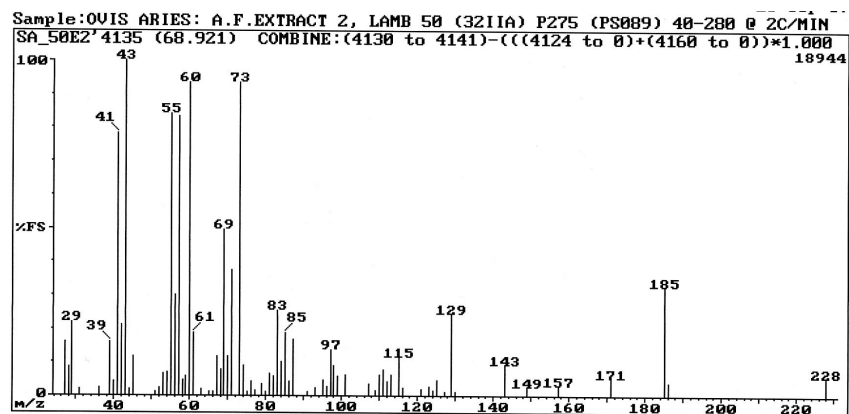


Fig. 3.42. The EI mass spectrum of 12-methyltridecanoic acid (C82).

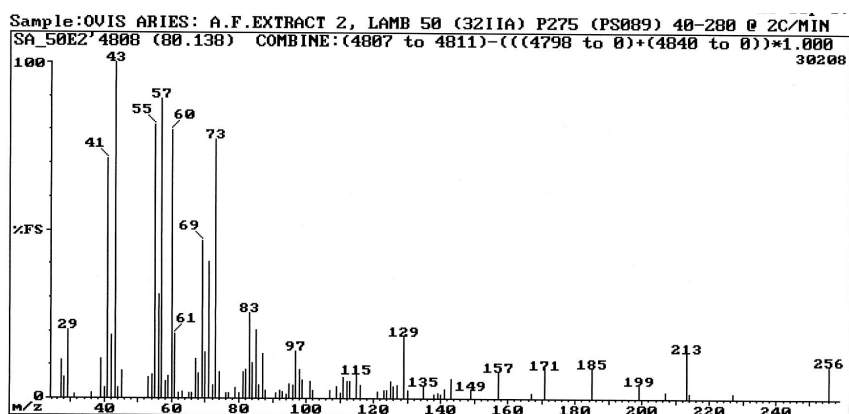


Fig. 3.43. The EI mass spectrum of 14-methylpentadecanoic acid (C102).

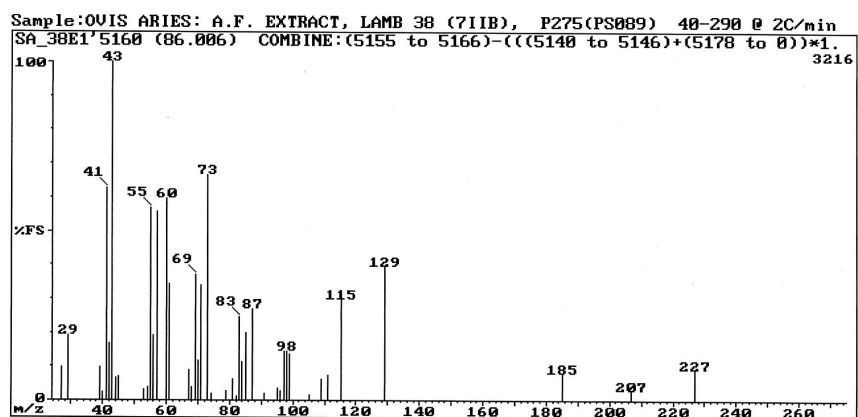


Fig. 3.44. The EI mass spectrum of 15-methylhexadecanoic acid (C106).

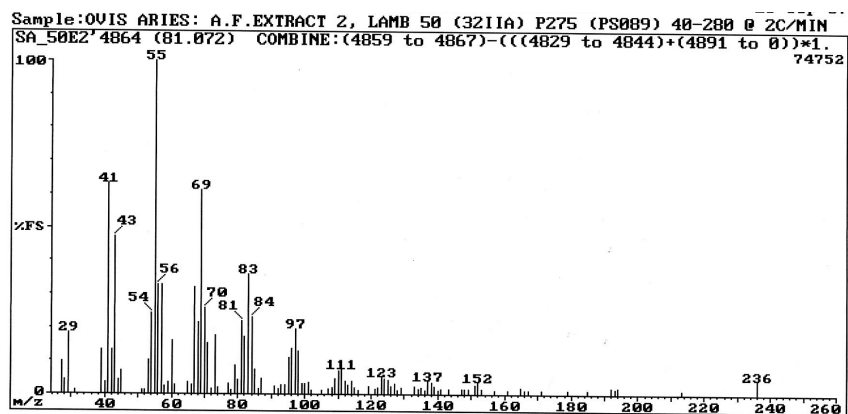


Fig. 3.45. The EI mass spectrum of (Z)-9-hexadecenoic acid (C103).

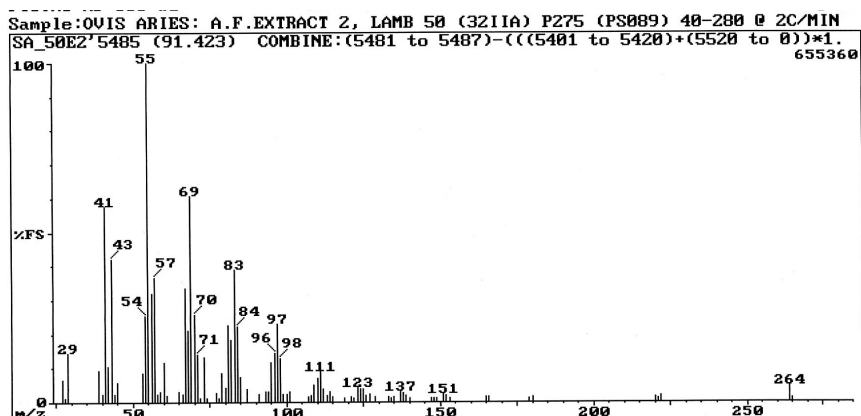


Fig. 3.46. The EI mass spectrum of (Z)-9-octadecenoic acid (C111).

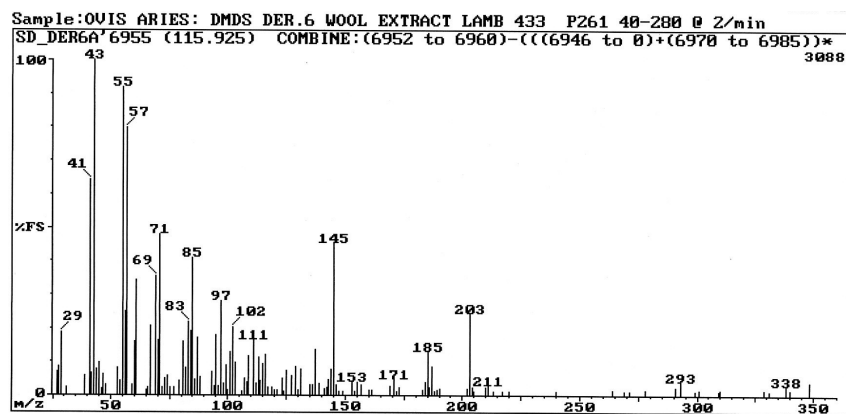


Fig. 3.47. The EI mass spectrum of DMDS adduct of (Z)-9-hexadecenoic acid (C103).

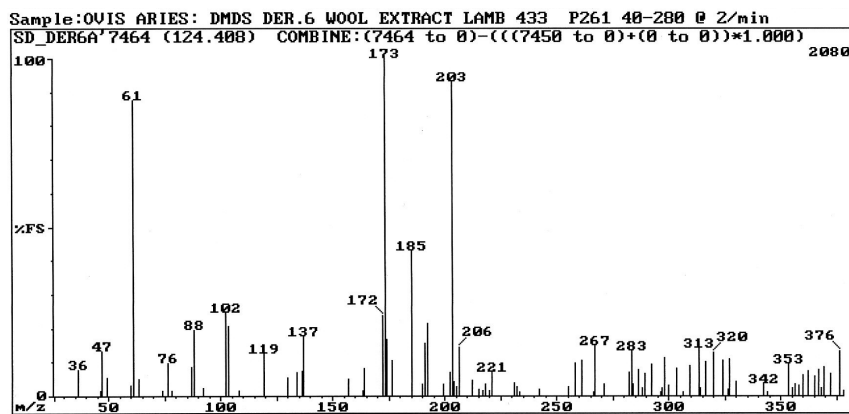


Fig. 3.48. The EI mass spectrum of DMDS adduct of (Z)-9-octadecenoic acid (C111).

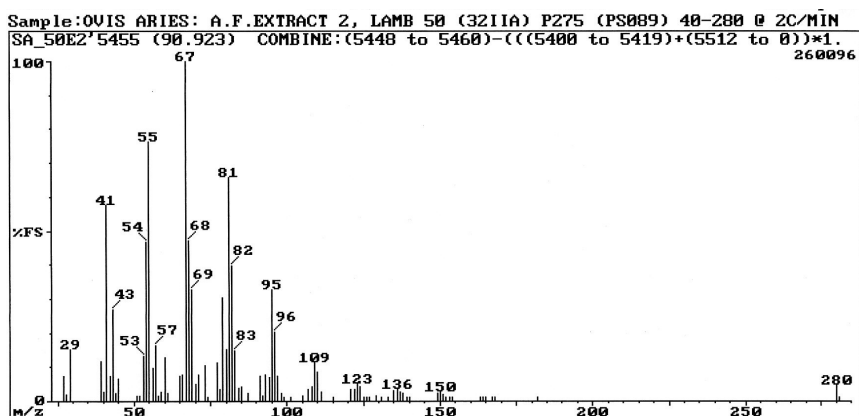


Fig. 3.49. The EI mass spectrum of (Z, Z)-9,12-octadecadienoic acid (C110).

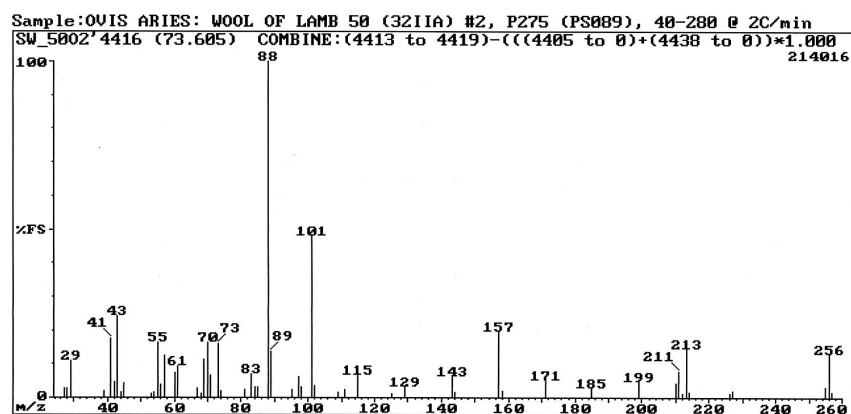


Fig. 3.50. The EI mass spectrum of ethyl tetradecanoate (C88).

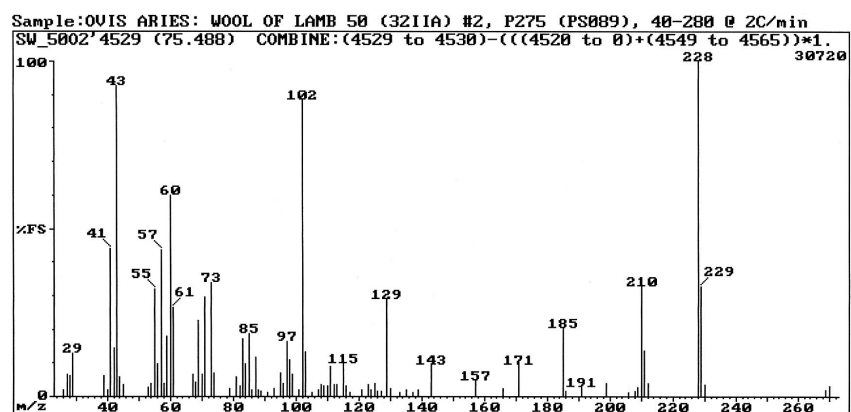


Fig. 3.51. The EI mass spectrum of isopropyl tetradecanoate (C92).

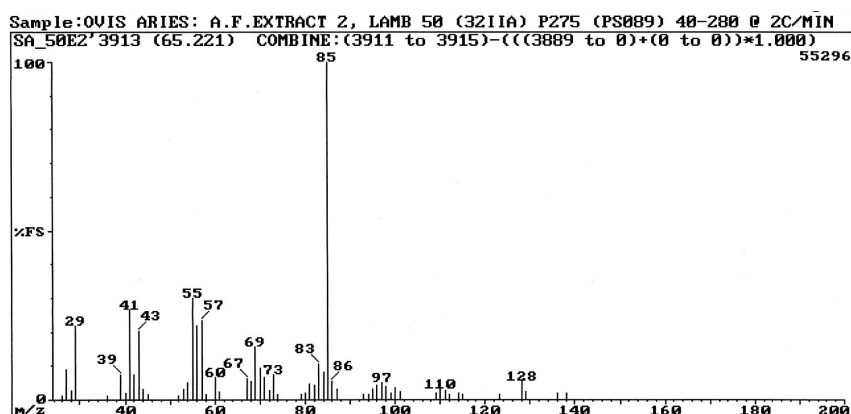


Fig. 3.52. The EI mass spectrum of dodecan-4-olide (C76).

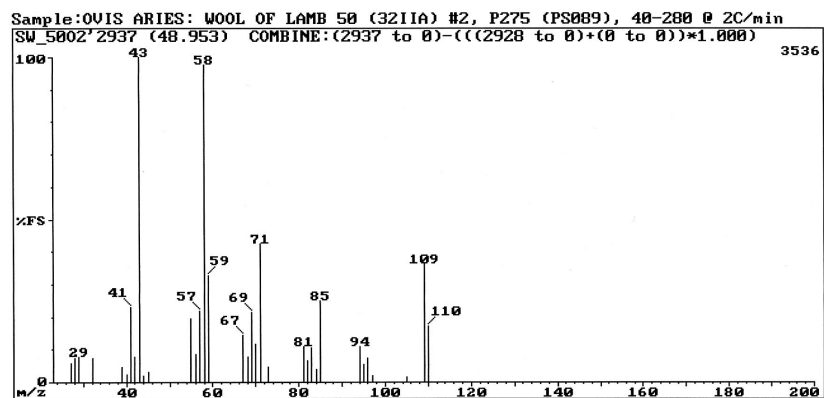


Fig. 3.53. The EI mass spectrum of 6,10-dimethyl-2-undecanone (C56).

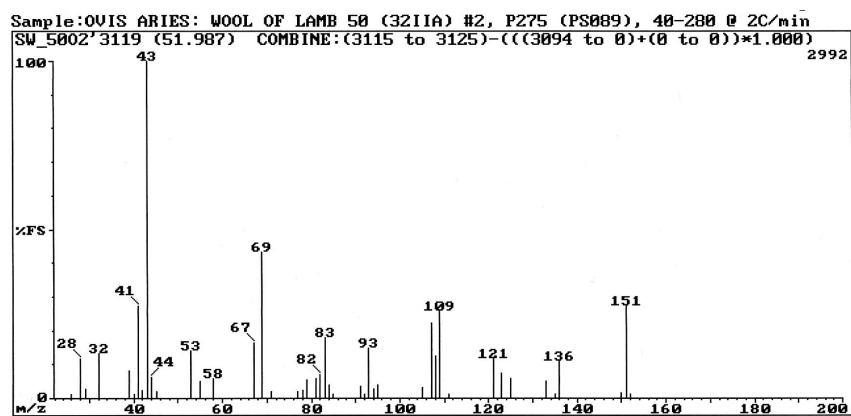


Fig. 3.54. The EI mass spectrum of (5E)-6,10-dimethyl-5,9-undecadien-2-one (C60).

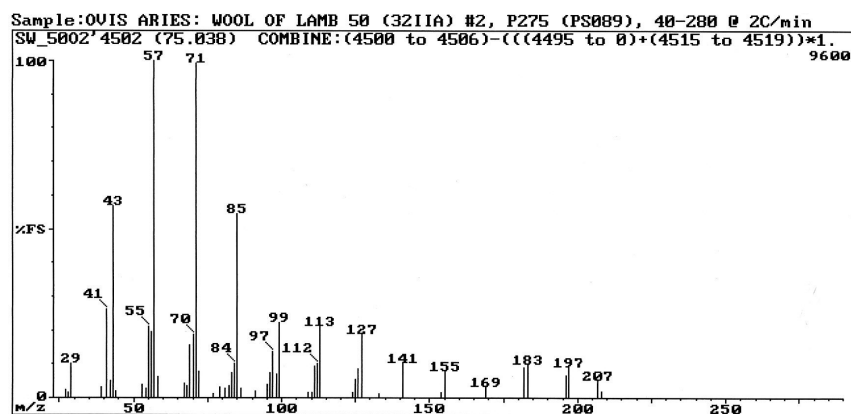


Fig. 3.55. The EI mass spectrum of 2,6,10,14-tetramethylhexadecane (C91).



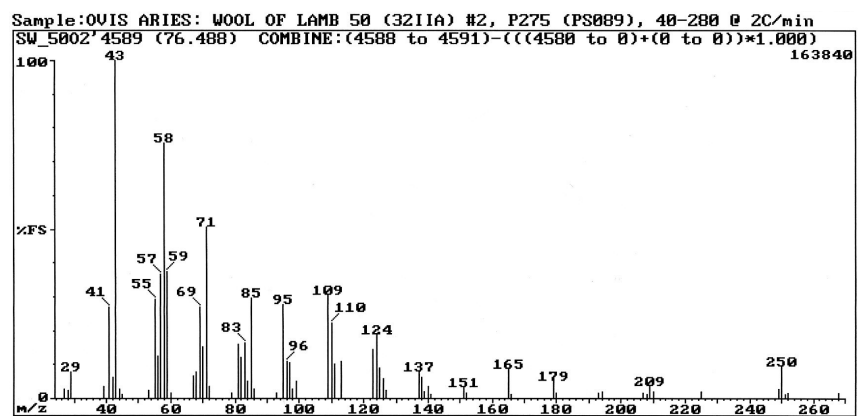


Fig. 3.56. The EI mass spectrum of 6,10,14-trimethyl-2-pentadecanone (C93).

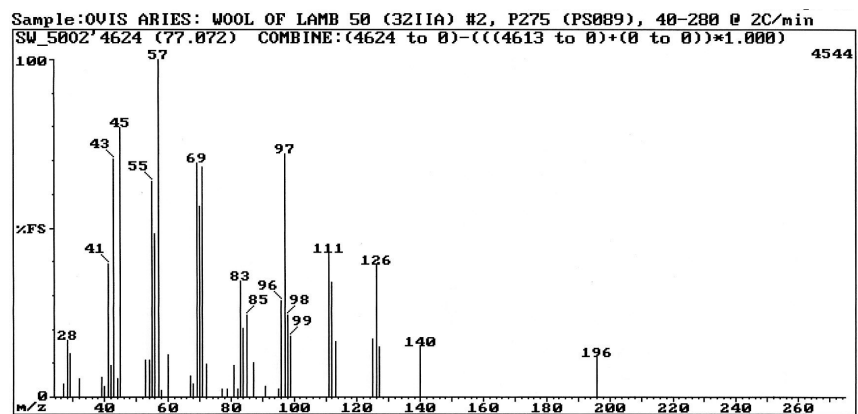


Fig. 3.57. The EI mass spectrum of 6,10,14-trimethyl-2-pentadecanol (C95).

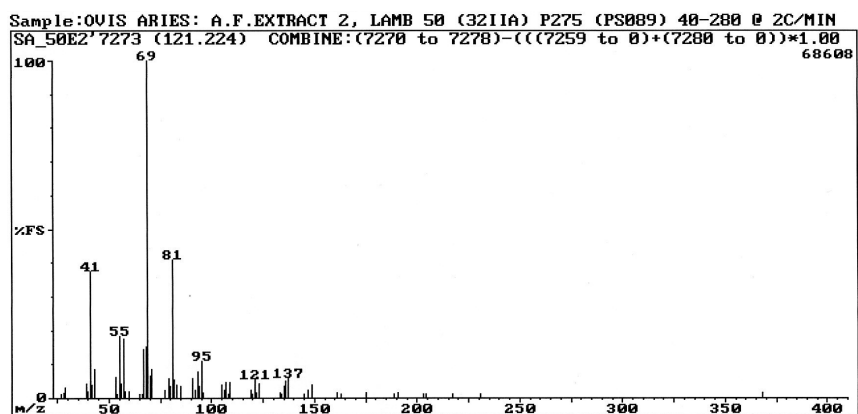


Fig. 3.58. The EI mass spectrum of squalene (C122).

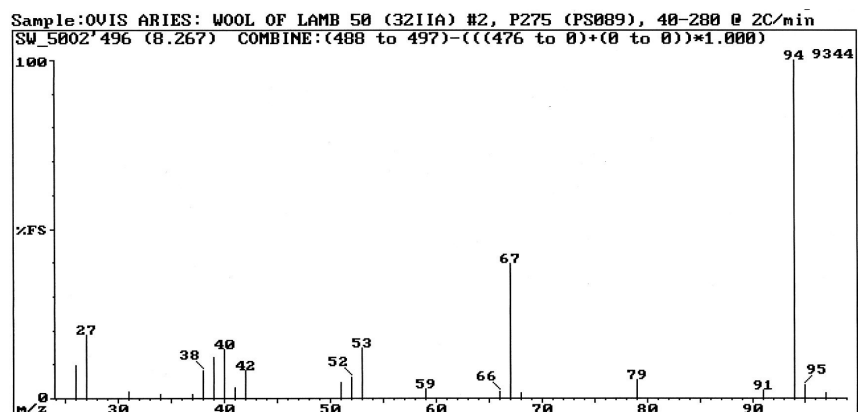


Fig. 3.59. The EI mass spectrum of 2-methylpyrimidine (C5).

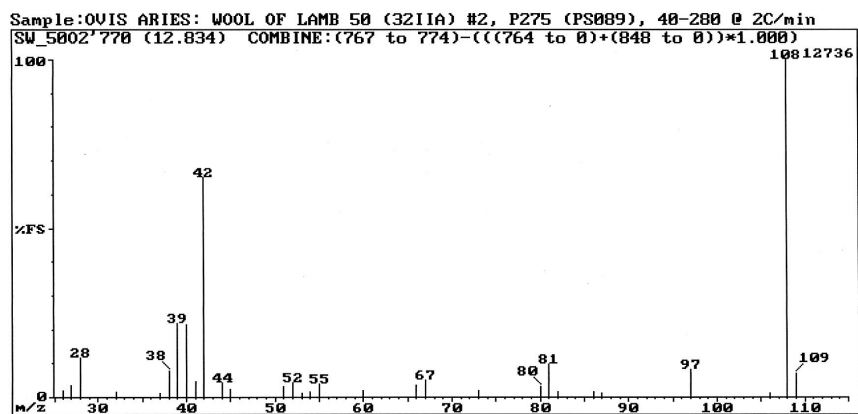


Fig. 3.60. The EI mass spectrum of 2,5-dimethylpyrimidine (C11).

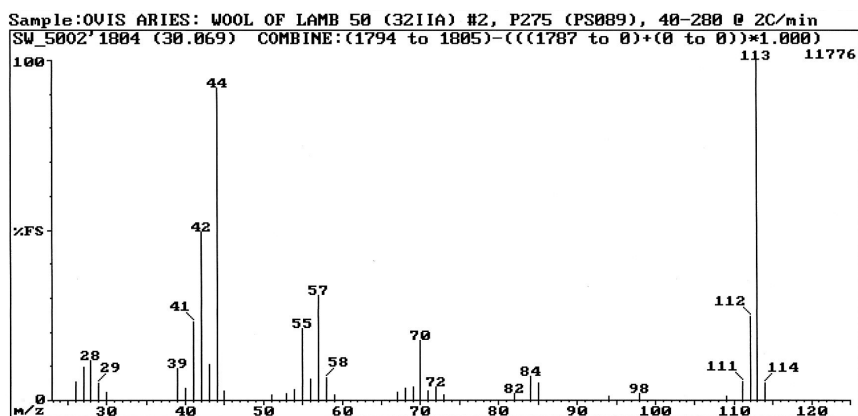


Fig. 3.67. The EI mass spectrum of N-methyl-2-piperidinone (C29).

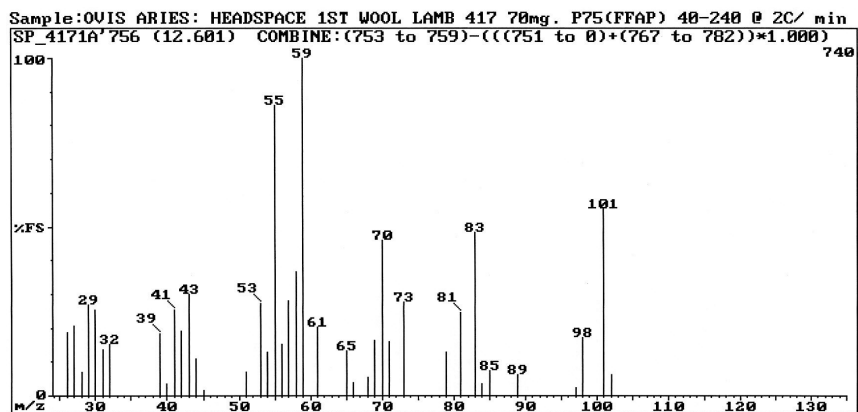


Fig. 3.68. The EI mass spectrum of 3-octanol (CP2).

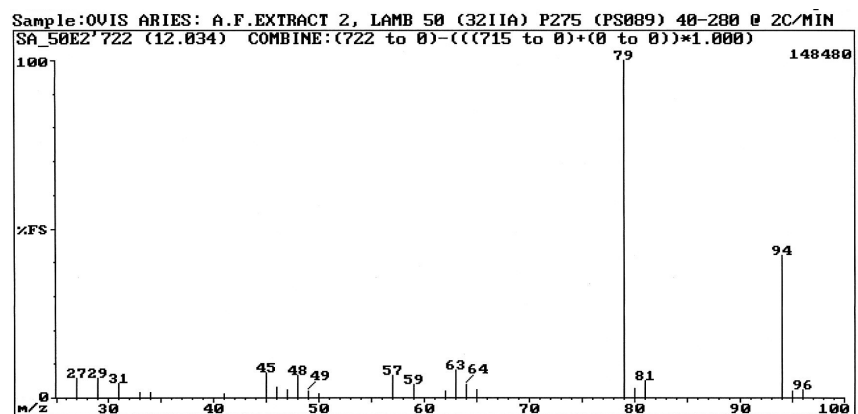


Fig. 3.69. The EI mass spectrum of dimethyl sulfone (C9).

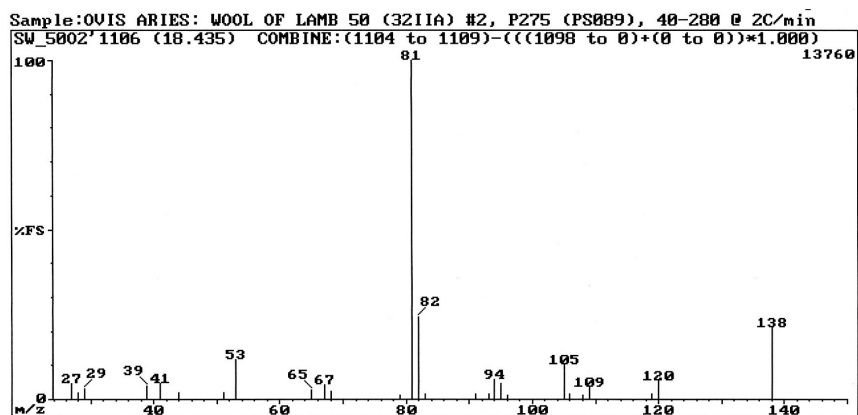


Fig. 3.70. The EI mass spectrum of 2-pentylfuran (C18).

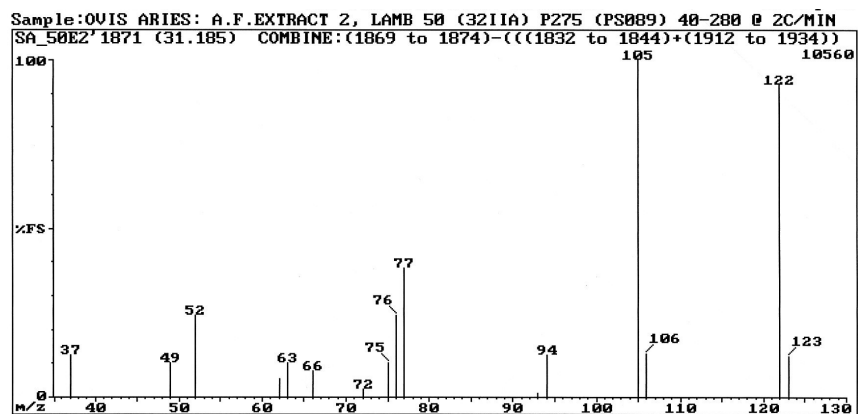


Fig. 3.71. The EI mass spectrum of benzoic acid (C32).

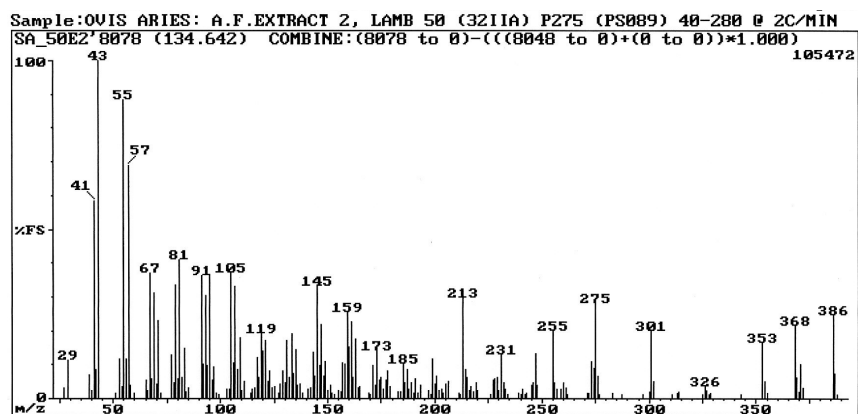
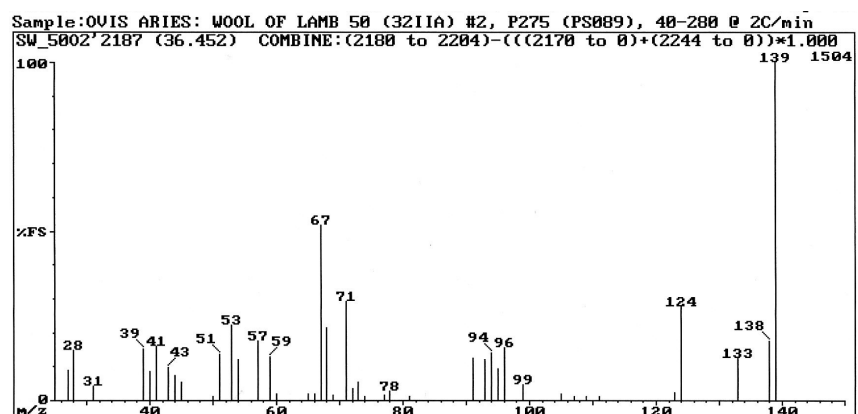
Fig. 3.72. The EI mass spectrum of cholest-5-en-3 $\beta$ -ol (C124).

Fig. 3.73. The EI mass spectrum of 3-ethyl-4-methyl-1H-pyrrole-2,5-dione (C37).

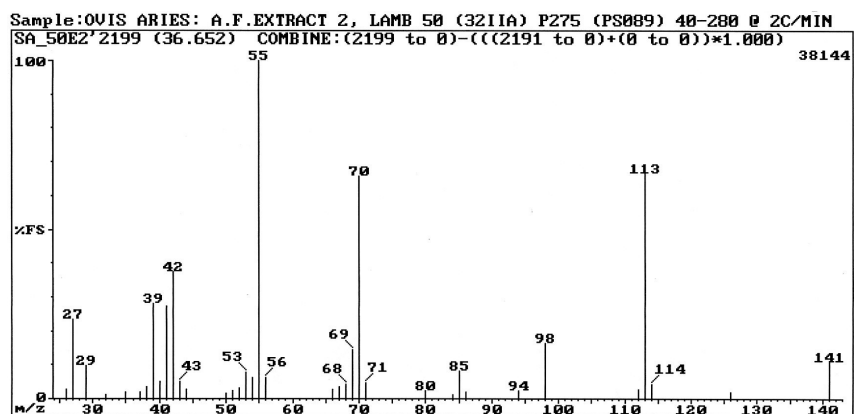


Fig. 3.74. The EI mass spectrum of 3-ethyl-4-methylpyrrolidine-2,5-dione (C38).

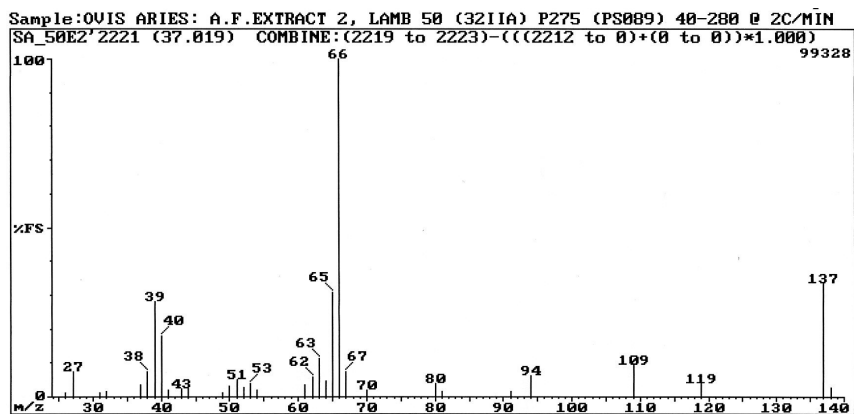


Fig. 3.75. The EI mass spectrum of 3-methyl-4-vinyl-1H-pyrrole-2,5-dione (C39).

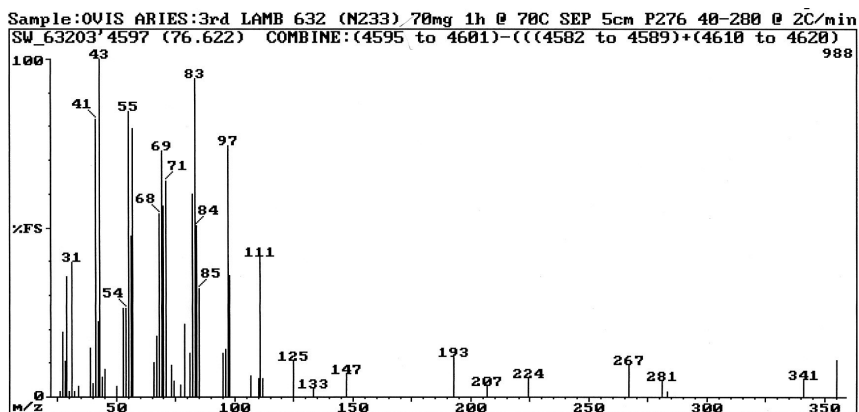


Fig. 3.76. The EI mass spectrum of unidentified constituent C96.

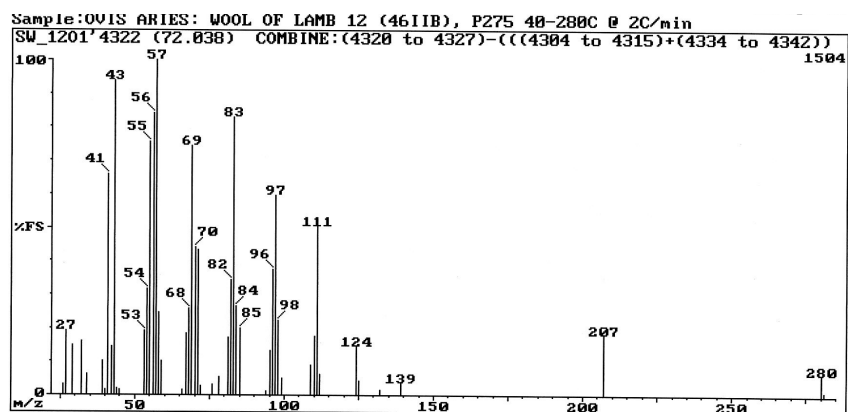


Fig. 3.77. The EI mass spectrum of unidentified constituent C84.

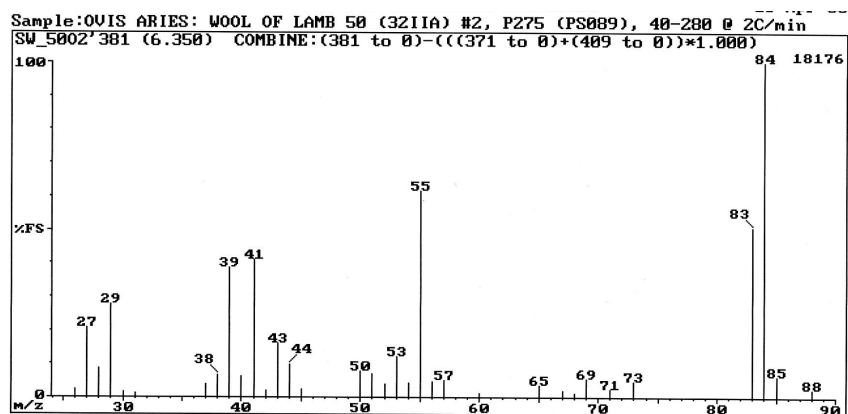


Fig. 3.78. The EI mass spectrum of unidentified constituent C3.

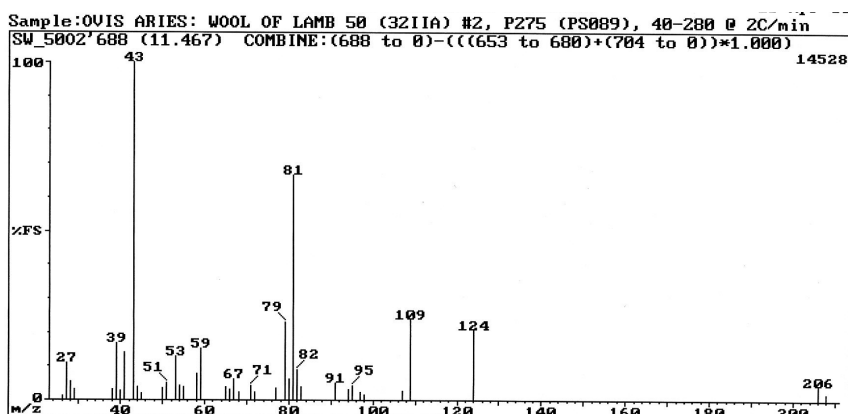
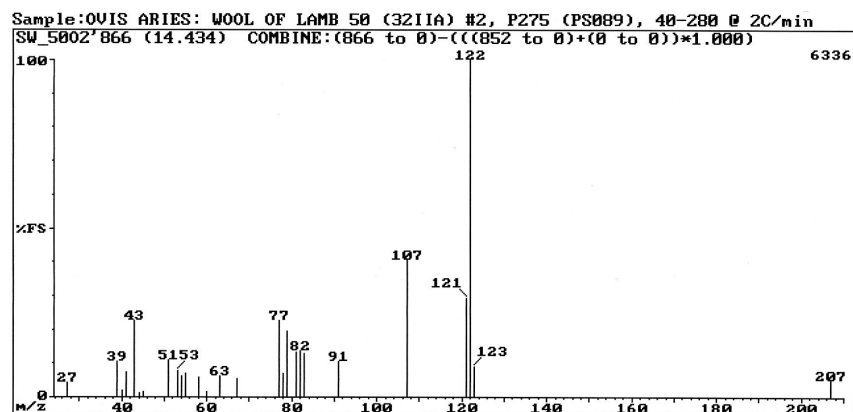
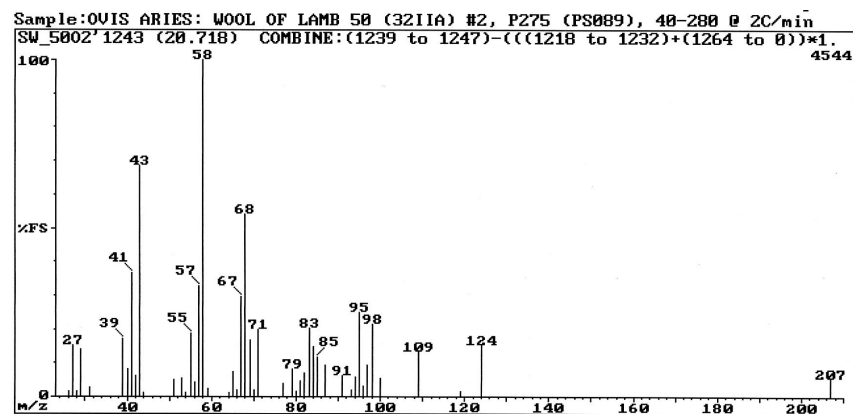
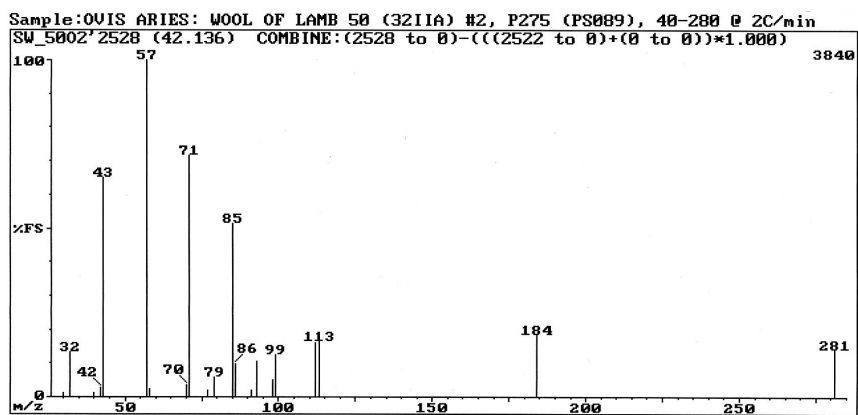


Fig. 3.79. The EI mass spectrum of unidentified constituent C7.

Fig. 3.80. The EI mass spectrum of unidentified constituent **C12**.Fig. 3.81. The EI mass spectrum of unidentified constituent **C21**.Fig. 3.82. The EI mass spectrum of unidentified constituent **C49**.

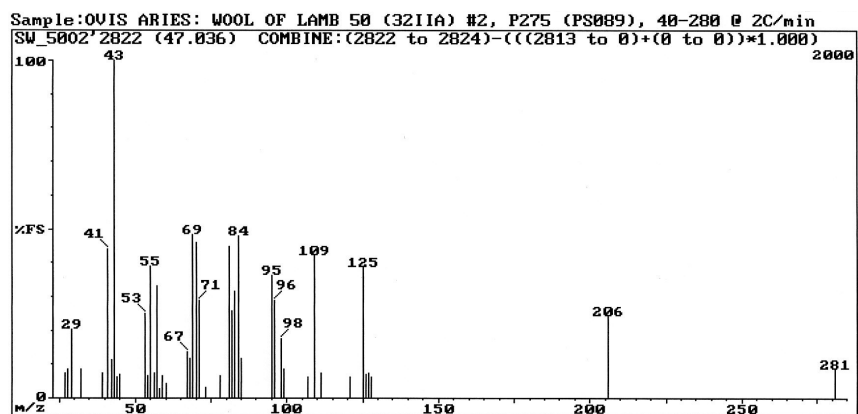


Fig. 3.83. The EI mass spectrum of unidentified constituent C52.

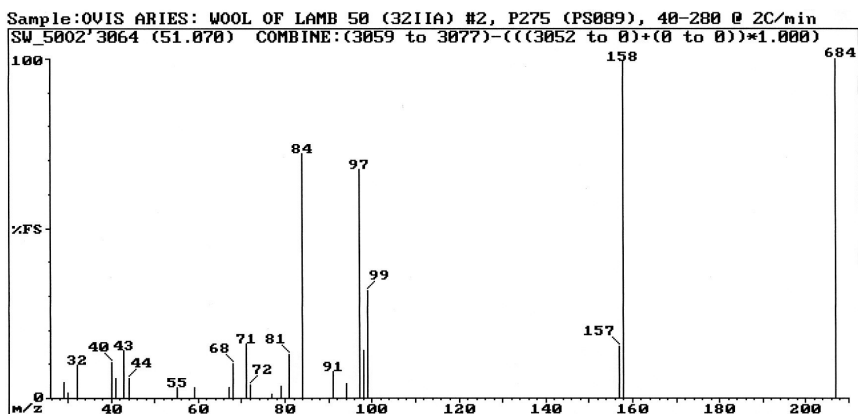


Fig. 3.84. The EI mass spectrum of unidentified constituent C59.

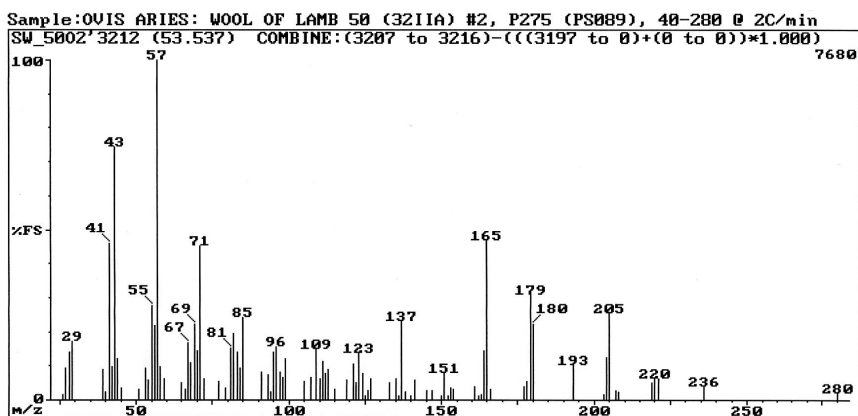


Fig. 3.85. The EI mass spectrum of unidentified constituent C63.



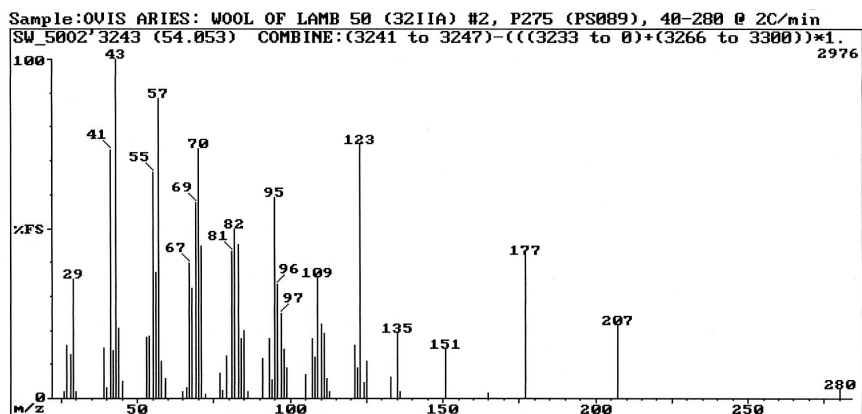


Fig. 3.86. The EI mass spectrum of unidentified constituent C64.

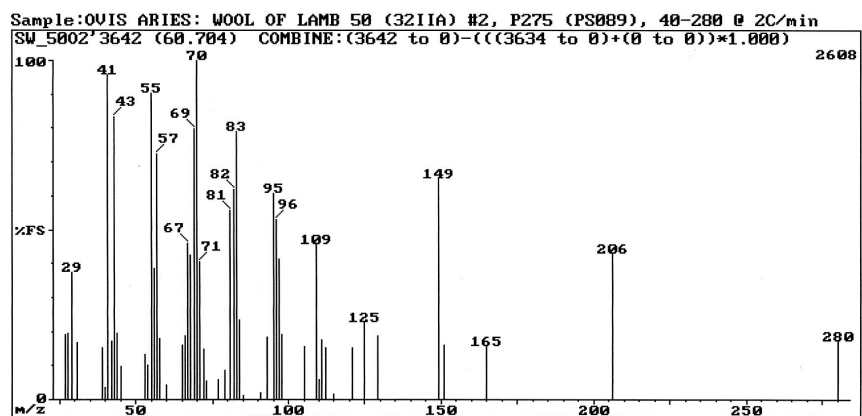


Fig. 3.87. The EI mass spectrum of unidentified constituent C70.

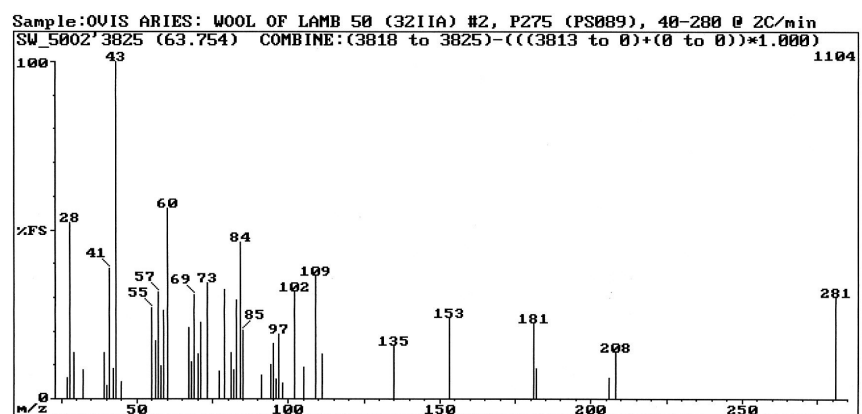


Fig. 3.88. The EI mass spectrum of unidentified constituent C74.

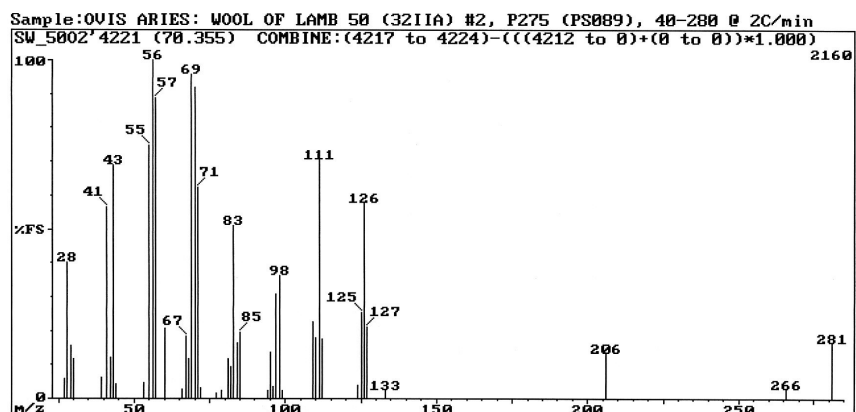


Fig. 3.89. The EI mass spectrum of unidentified constituent C83.

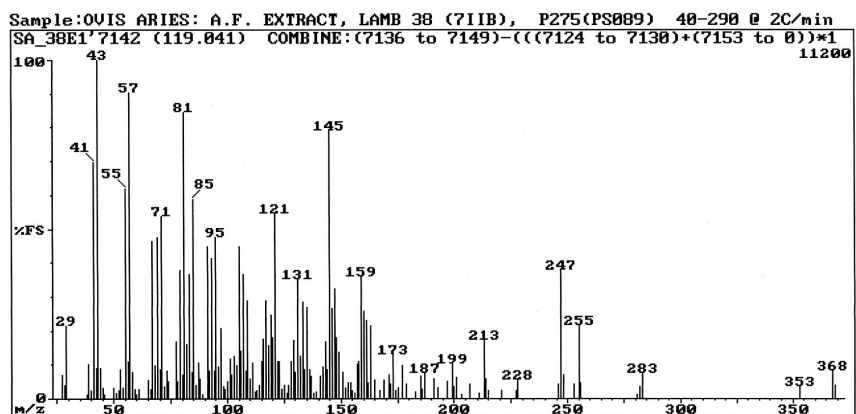


Fig. 3.90. The EI mass spectrum of unidentified constituent C121.

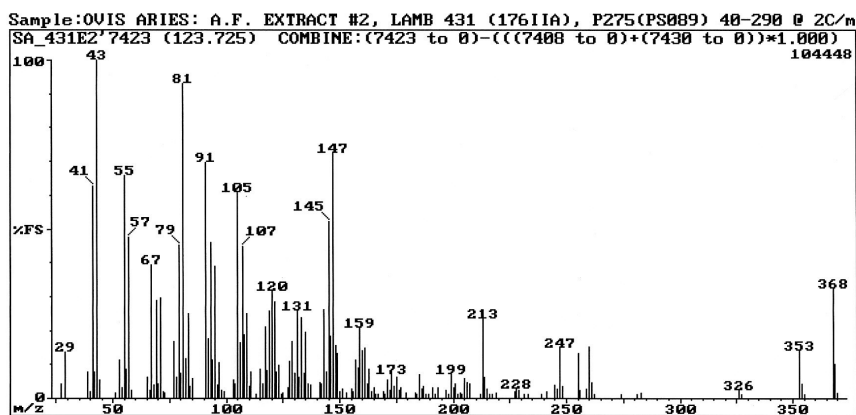


Fig. 3.91. The EI mass spectrum of unidentified constituent C123.

## CHAPTER 4

# QUANTITATIVE ANALYSIS OF COMPOUNDS PRESENT IN THE AMNIOTIC FLUID OF DÖHNE MERINO EWES AND IN THE WOOL OF THEIR LAMBS

### 4.1 Quantitative Analysis of Volatile Organic Fraction of the Wool Samples

#### 4.1.1 Introduction

In the first part of this chapter the determination of the quantitative composition of the volatile fraction of wool of twin lambs and the statistical analysis of the quantitative results will be discussed. Wool samples were collected from the forehead of each of the 16 lambs in the sample group (Plate 2.1c) the morning after birth and again seven days later from five twins (10 lambs). Five of the ewes that were used in the sample group during the 2007 lambing season fortunately also had lambs two years later and these lambs were used for further analytical work during the lambing season of 2009. The following three aspects of kin recognition in sheep were investigated:

1. The genetic influence on the odour profile of lambs was investigated by intra-twin comparison of the qualitative and quantitative composition of the wool volatiles of twin lambs and inter-twin comparison of the wool volatiles of randomly selected lambs, which amounted to comparing the intra-twin similarities with the inter-twin differences in the composition of the wool volatiles. Romeyer *et al.* (1993) showed that ewes use phenotype matching to recognise their lambs, since recognition was not influenced when the genetic make-up of the ewes differed from that of the lambs, neither was recognition influenced by the ewe's secretions. A ewe was implanted with monozygotic twin embryos. After birth she was allowed to learn the odour of one of the twin lambs. When presented with the other twin, she could recognise the second monozygotic twin lamb without being previously exposed to this lamb. However, when this experiment was repeated with dizygotic twin lambs, the ewe could not identify the non-identical twin lamb after being exposed to the other dizygotic twin lamb. This experiment shows that at least some of the features of the

lamb's odour is genetically based (Wyatt, 2003: 109–110). A record of the breeding programme of the flock of sheep at Mariendahl experimental farm is maintained in a computerised flock-recording program (Olivier, 2003). This record made it possible to study the above-mentioned genetic influences on the composition of the putative semiochemicals by comparing the intra-twin and inter-twin similarities or differences in the composition of the wool volatiles of twins and unrelated lambs, respectively.

The breeding programme at Mariendahl did however not allow for the use of exactly the same sample group during the lambing seasons of 2007 and 2009. Nonetheless, although the sample group was considerably reduced to five ewes in 2009, the information gained from investigating the odour profiles of lambs born of the same ewe two years later provided valuable insights into how odour is linked to genetic trends. In other words, it was possible to find out whether lambs born during 2007 and 2009 to the same ewe possess odour profiles that are more similar than the odour profiles of any randomly selected non-twin lambs born during these two years.

2. By collecting the wool samples the morning after birth and again seven days later, changes in the odour profile of lambs could be traced. In this manner it could be determined whether the odour of each lamb is fixed, unaffected by time or environmental factors, or if the odour is continually changing, which would compel the ewe to continue learning the odour of her lamb, *i.e.* to adapt her olfactory system to the odour changes during the period in which olfactory recognition is the main mode of identification of the lamb.
3. The odour profiles of wool samples collected from a ewe and from her lamb were compared and qualitative and quantitative similarities assessed to determine whether maternal labels are possibly used in the pheromone communication between lamb and ewe.

Information pertaining to the lambs studied during the lambing seasons of 2007 and 2009 is summarised in Table 4.1.

**Table 4.1: Ewe and lamb sample groups of 2007 and 2009**

Lamb US number	Sex	Birth order	Parents of the lamb	
			Ewe US number	Ram US number
<b>Lambs born during the lambing season of 2007</b>				
US-2007-0011	Female	1		
US-2007-0012	Female	2	US-2004-0463	XP-2005-0234
US-2007-038	Female	2		
US-2007-039	Female	1	US-2001-0048	XP-2005-0234
US-2007-049	Female	2		
US-2007-050	Male	1	US-2003-0202	XP-2005-0234
US-2007-0103	Female	1		
US-2007-0104	Male	2	US-2002-0424	MR-2005-0099
US-2007-0105	Female	2		
US-2007-0106	Male	1	US-2003-0219	MR-2005-0099
US-2007-0114 <sup>a</sup>	Female	2		
US-2007-0116 <sup>a</sup>	Female	1	US-2001-0739	MR-2005-0099
US-2007-0224	Female	1		
US-2007-0225	Male	2	US-2004-0517	US-2004-0321
US-2007-0406	Male	1		
US-2007-0407	Male	2	US-2003-0509	US-2005-0115
US-2007-0408	Female	2		
US-2007-0409	Male	1	US-1999-0134	US-2005-0115
US-2007-0417	Male	1		
US-2007-0418	Male	2	US-2001-0251	US-2005-0115
US-2007-0431	Female	1		
US-2007-0432	Female	2	US-1999-0520	US-2005-0115
US-2007-0433	Female	1		
US-2007-0434	Male	2	US-2000-0633	US-2005-0115
US-2007-0516	Male	1		
US-2007-0517	Male	2	US-2001-740	US-2005-0212
US-2007-0604	Male	1		
US-2007-0605	Male	2	US-2002-0325	US-2005-0235
US-2007-0624	Female	1		
US-2007-0625	Female	2	US-2005-0611	US-2005-0235
US-2007-0637	Female	1		
US-2007-0638	Male	2	US-2004-0314	US-2005-0235
<b>Lambs born during the lambing season of 2009<sup>b</sup></b>				
US-2009-0330	Female		US-2002-0424	US-2006-0550
US-2009-0520	Female			
US-2009-0521	Female		US-2004-0314	US-2007-0100
US-2009-0541	Female			
US-2009-0542	Male		US-2003-0509	US-2007-0100
US-2009-0548	Female		US-2003-0202	US-2007-0100
US-2009-0632	Female			
US-2009-0633	Male		US-2003-0219	US-2007-0130
US-2009-0736	Female		US-2006-0352	US-2007-0425

<sup>a</sup> These numbers were allocated to the first- and second-born lambs of a triplet. Only first- and second-born lambs were used in this study.

<sup>b</sup> The birth order was not of interest during the investigation into the qualitative and quantitative composition of the wool in 2009, and this information is not included in the table.

The many compounds identified in this study were present in at least 10% of the wool samples investigated during the lambing season of 2007. This feature of this dataset is in agreement with the constraints on the design of chemical communication systems used for kin recognition, which, according to Alberts (1992), requires a wide variety of compounds present in different concentrations.

#### 4.1.2 Quantitative composition

Headspace analyses of the cranial wool of the lambs were carried out to determine the relative quantitative composition of the wool volatiles. Different methods of quantification were compared.

Quantitative data obtained from GC-MS analyses are normally less reliable than data obtained from GC-analyses. However, due to the inherent qualitative unreliability of GC data when it comes to the assignment of peak identity in complex chromatograms, the integration of data obtained in GC-MS analyses was preferred. In this regard, it must also be taken into account that in LECUS hydrogen is used as carrier gas for GC analyses and helium as carrier gas for GC-MS work. It is known that the elution order of compounds belonging to different compound classes could change when changing from one carrier gas another. As far as quantification is concerned, the following procedures were followed: GC- as well as GC-MS analyses were carried out on extracts of the same wool samples that were also analysed by SEP-GC-MS in order to correct for differences in the molar FID and MS responses of the analytes, at least as far as the major, easily identifiable constituents are concerned. In the analyses of the extracts, the internal standard (IS) method, with methyl hexanoate, methyl undecanoate and methyl hexadecanoate as internal standards, was used for quantification. For various reasons the results that were obtained in this manner were not particularly reliable. However, when the quantitative work was done, synthetic analogues of practically all of the VOCs identified in the collected samples were available. Results that are more reliable were obtained with less risk of errors by simply using the available synthetic compounds as external standards for the quantification of the VOCs in the headspace of wool samples. As regards the reliability of the quantitative data obtained by SEP-GC-MS analyses, RSDs of better than 5% are normally obtained with SEPs of the second generation that were used in this study (unpublished results, Burger *et al.*).

Although the relative peak areas were used in statistical analyses, these quantitative analyses were done merely to obtain an approximate idea of the quantities of the VOCs present in the headspace of wool samples.

To keep the very large volume of data within reasonable limits, average masses (ng) of the volatiles present in all 32 wool samples are tabulated in Table 4.3. The compounds that are present in Table 3.1 but not in Table 4.3 were identified by GC-HRMS analysis. They were not observed in the analyses done with GC-LRMS using column A and therefore they could not be quantified relative to the other compounds. In all graphs discussed below the relative quantity of each constituent is displayed as an average percentage peak area. Constituents present in concentrations of less than 0.001% of the total peak area were not considered in this investigation. The graphic presentation of the data does not include compounds that were only identified by GC-HRMS analysis of the wool samples. The polar column B was only used in GC-MS analyses of wool samples collected during the lambing season of 2007, *i.e.* not in analyses of the wool samples collected during the lambing season of 2009. The constituents identified in 2007 using column B are thus not included in the bar graphs in which the results of the quantitative analyses of the wool extracts of 2007 are compared with those of 2009 (Figs. 4.3 to 4.5). The error bars shown in the graphs represent the standard error of the mean (SEM):

$$\text{SEM}_x = \frac{\sigma}{\sqrt{n}}$$

where  $\sigma$  is the sample standard deviation and  $n$  the sample size or, in this case, the number of observations of the constituent in the respective wool samples.

The standard deviation ( $\sigma$ ) measures the variability that can be expected between individuals. In determining whether an observed mean is different from a standard, the SEM includes the influences of variability of the observations, as well as the size of the samples investigated (Dawson-Saunders and Trapp, 1994: 85–87, 102), making the SEM better suited for the current investigation, given that all the constituents were not always present in all the analysed wool samples

#### 4.1.2.1 Wool from day-old lambs

As mentioned in § 3.1.5, of the 87 constituents identified in the wool collected during the lambing season of 2007, 14 constituents were not found in the wool collected

during the lambing season of 2009. A total of 10 constituents identified in the wool collected during the lambing season of 2009 were not identified in the wool collected during the lambing season of 2007. Of these 10 new constituents, five were also identified in the amniotic fluid collected in 2007 (Table 3.1). This indicates that more or less the same constituents are always used for semiochemical communication in this flock of sheep. In the discussion that follows, an attempt is made to compare the quantitative compositions of the volatile fractions of wool samples collected during the lambing seasons of 2007 and 2009. As a first approximation, the average quantities expressed as percentage peak areas obtained in all the analyses of these samples collected during both lambing seasons are used because only five of the 16 ewes were in the experimental groups of both years.

Approximately 49% of the total percentage average peak area of the volatile compounds present in the headspace of the wool of day-old Döhne Merino lambs born during the lambing season of 2007, and taken as an average value for the 32 samples analysed, comprise of the following five constituents: ethyl tetradecanoate (**C88**) (20%), nonanal (**C27**) (12%), 6,10,14-trimethyl-2-pentadecanone (**C93**) (9%), tetradecanal (**C73**) (4%) and pentadecanal (**C81**) (4%) (Fig. 4.1).

When comparing the major volatile compounds (in terms of percentage peak areas) identified in the wool of day-old lambs born during the lambing season of 2007 with the major volatile compounds identified in the wool of day-old lambs born during the lambing season of 2009, a slightly different pattern is seen (Fig. 4.2). Approximately 38% of the total amount of the volatile compounds present in the headspace of the wool of day-old lambs born during the lambing season of 2009 comprise of the following four constituents: ethyl tetradecanoate (**C88**) (18%), nonanal (**C27**) (10%), 6,10,14-trimethyl-2-pentadecanone (**C93**) (5%) and hexadecanoic acid (**C104**) (5%).



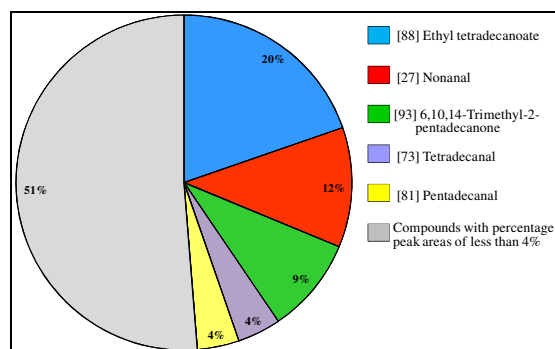


Fig. 4.1. Quantitative composition of the wool volatiles collected from day-old Döhne Merino lambs during the lambing season of 2007.

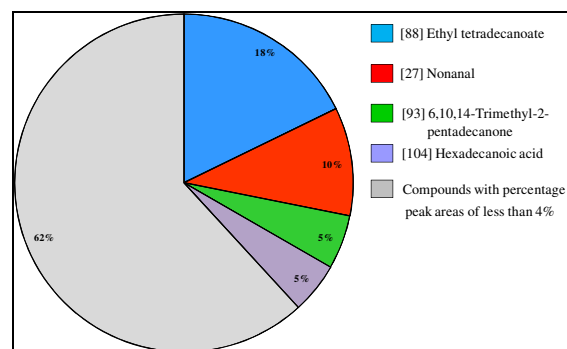


Fig. 4.2. Quantitative composition of the wool volatiles collected from day-old Döhne Merino lambs during the lambing season of 2009.

Ethyl tetradecanoate, nonanal and 6,10,14-trimethyl-2-pentadecanone are major constituents of the wool collected from lambs born during 2007 and in 2009. The two long-chain aldehydes, tetradecanal and pentadecanal, have lower percentage peak areas in 2009 than in 2007. Hexadecanoic acid has previously been identified as a constituent of lanolin (Table 3.1). Other than hexadecanoic acid none of these major wool volatiles have previously been identified in lanolin or in the wool of sheep (Lisovac and Shooter, 2003; Motiuk, 1979a, 1979b, 1980; Schlossman and McCarthy, 1979)

The majority of the compounds identified in the wool collected from day-old lambs born during the lambing seasons of both 2007 and 2009 are present in relatively low concentrations; percentage peak areas are less than 4% of the total peak area of all the constituents (Figs. 4.1 and 4.2).

The constituents identified in the wool of day-old lambs born during the lambing season of 2007 and 2009 in terms of percentage peak areas larger than 1% of the total are shown in Fig. 4.3. Most of the compounds in this figure show only relatively small variations and, surprisingly, in the light of the inherent inaccuracies of headspace analyses of volatile compounds, some of the constituents showing the smallest variations are the small molecules heptanal (C8), 6-methyl-2-heptanone (C14), octanal (C19) and hexanal (C4). The carboxylic acids (C69, C104), the ethyl- and isopropyl esters (C88, C92), pentadecanal (C81) and 6,10,14-trimethyl-2-pentadecanone (C93) display the largest variations in percentage peak areas. These variations could be attributed to small variations in sample collection, sample preparation and other operational procedures, although this is unlikely in the light of the remarkably good reproducibility of the

---

---

analyses of material from twins, *vidi infra*. However, considering that these results were obtained from samples collected in different lambing seasons, the small variations in the percentage peak areas of the identified compounds are quite remarkable. On the other hand, the constituents present in percentage peak areas smaller than 1% of the total show larger differences in a comparison of the average quantitative results obtained in 2007 and 2009 (Fig. 4.4).

These compounds and the quantitative composition of the wool volatiles are tabulated in Table. 4.3. The compounds showing the largest variation are discussed in § 4.1.3.

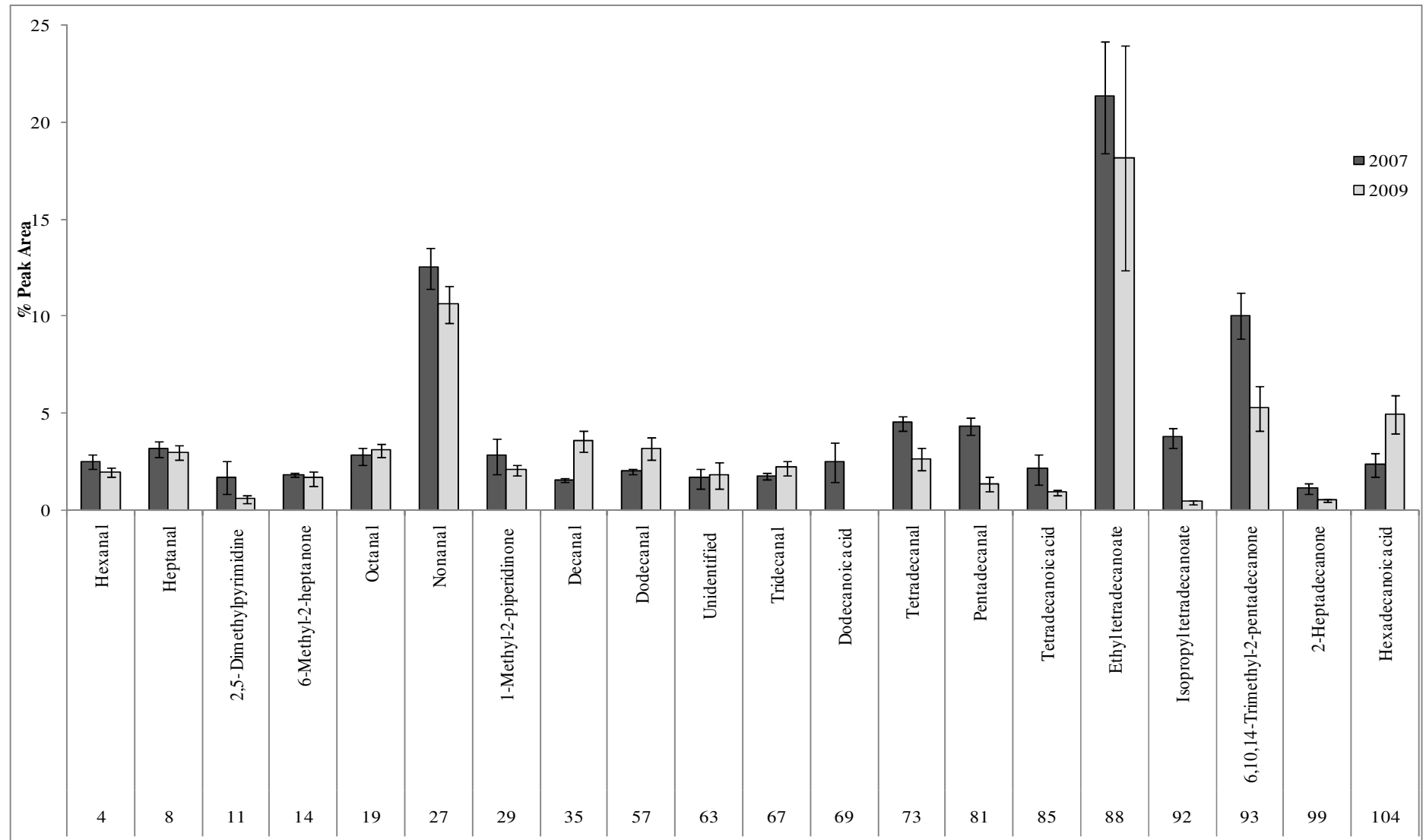


Fig. 4.3. Volatile constituents present in percentages larger than 1% in the wool of day-old lambs born during the lambing seasons of 2007 (dark grey) and 2009 (light grey). The constituents are numbered on the x-axis.

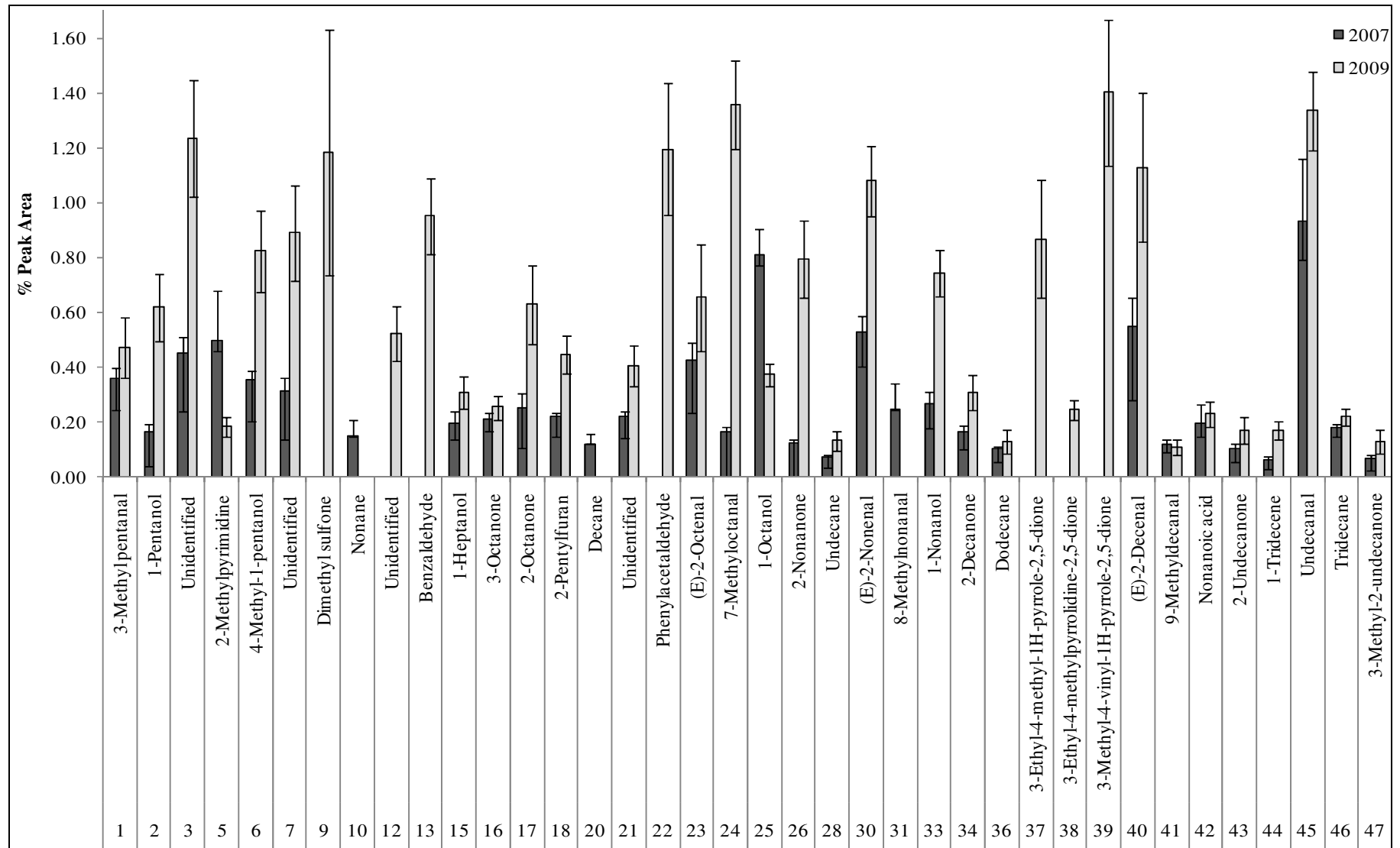


Fig. 4.4. Volatile constituents present in percentages smaller than 1% in the wool of day-old lambs born during the lambing seasons of 2007 (dark grey) and 2009 (light grey). The constituents are numbered on the x-axis. Part 1 of 2.

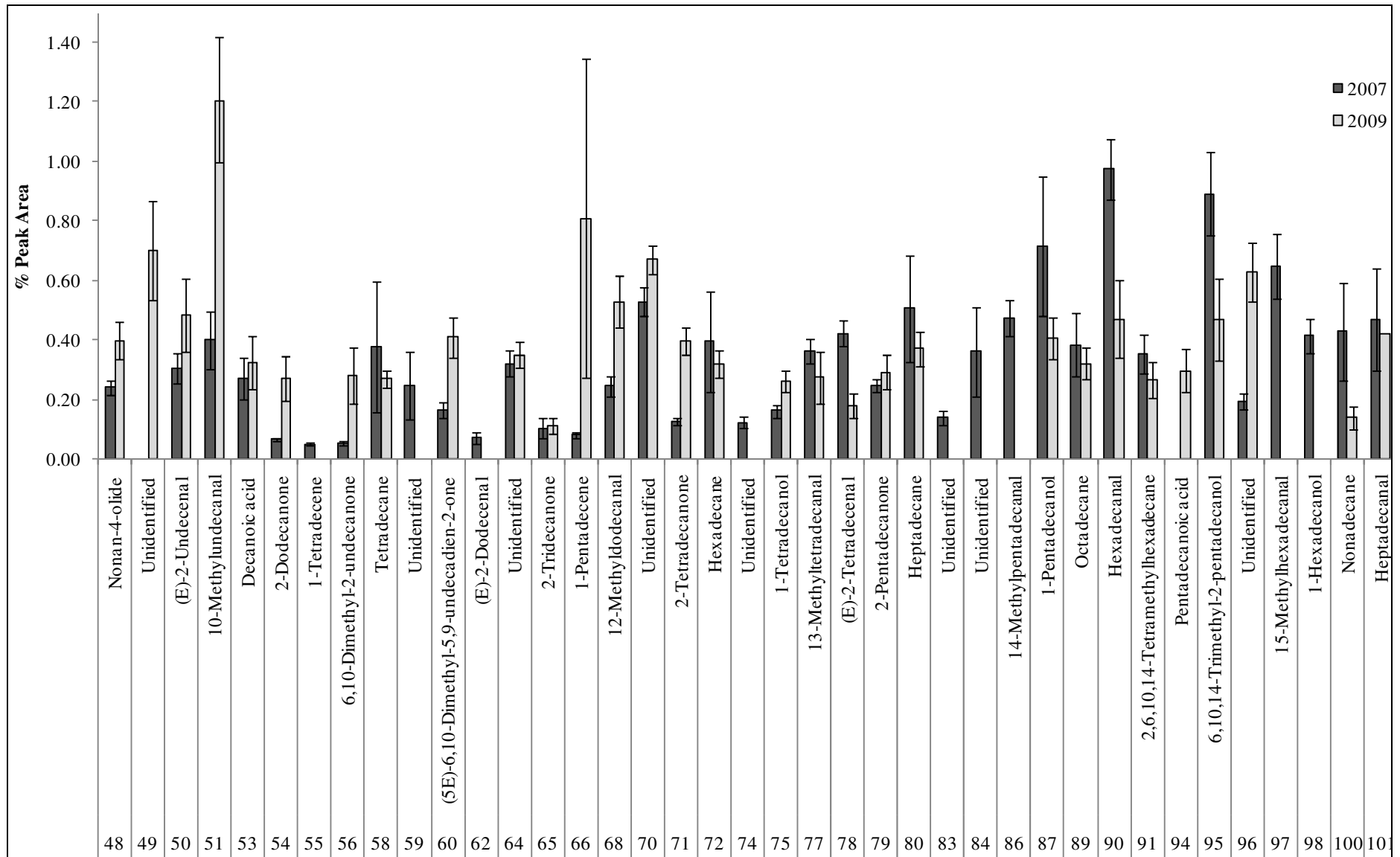


Fig. 4.4 Part 2 of 2.

The TICs of the wool volatiles of twin lambs were surprisingly similar. When, for example, the TICs of twin lambs US-2007-0105 and US-2007-0106 are compared with those of another set of twins US-2007-0114 and US-2007-0115 the intra-twin similarities in the TICs of one twin, and the inter-twin differences in the TICs of unrelated lambs, are obvious (Figs. 4.5 to 4.6).

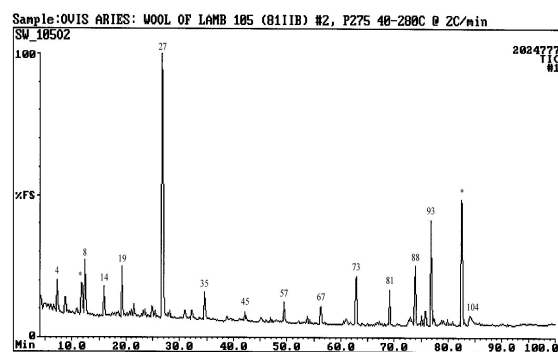


Fig. 4.5. TIC obtained by GC-MS analysis of the headspace of the wool of Döhne Merino lamb US-2007-0105.

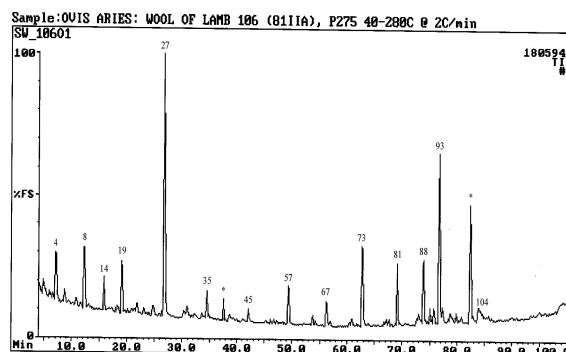


Fig. 4.6. TIC obtained by GC-MS analysis of the headspace of the wool of Döhne Merino lamb US-2007-0106.

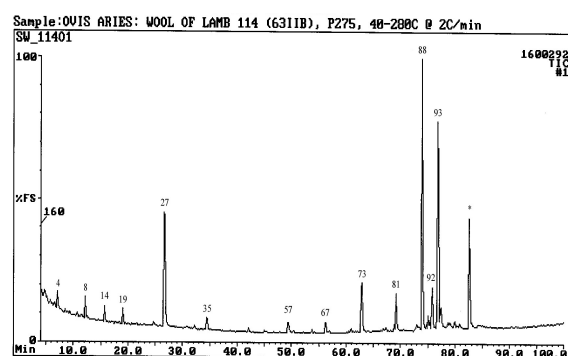


Fig. 4.7. TIC obtained by GC-MS analysis of the headspace of the wool of Döhne Merino lamb US-2007-0114.

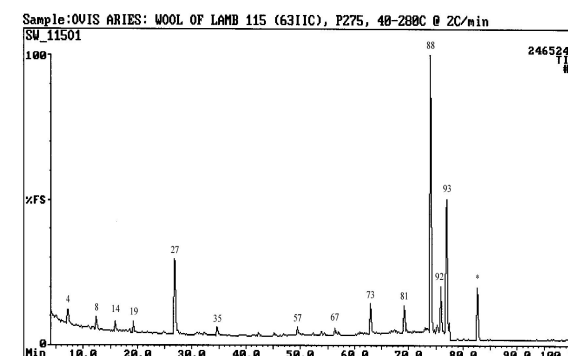


Fig. 4.8. TIC obtained by GC-MS analysis of the headspace of the wool of Döhne Merino lamb US-2007-0115.

In Fig. 4.9 the percentage peak areas obtained in SEP analyses of the cranial wool of the twin lambs US-2007-0224 and US-2007-0225 are superimposed. The similarity in the odour profiles is obvious. The concentrations of ethyl tetradecanoate (**C88**), 6,10,14-trimethyl-2-pentadecanone (**C93**) and hexadecanoic acid (**C104**) in the wool of the twin are so similar that the black trace representing the results for lamb US-2007-0224 is only visible at the apexes of the peaks of these compounds. The percentage peak areas for lamb US-2007-0224, displayed in Fig. 4.9, are compared in Fig. 4.10 with the percentage

peak areas obtained for the unrelated lamb US-2007-0050. Here the differences in the composition of the wool volatiles of these two lambs are also quite obvious.

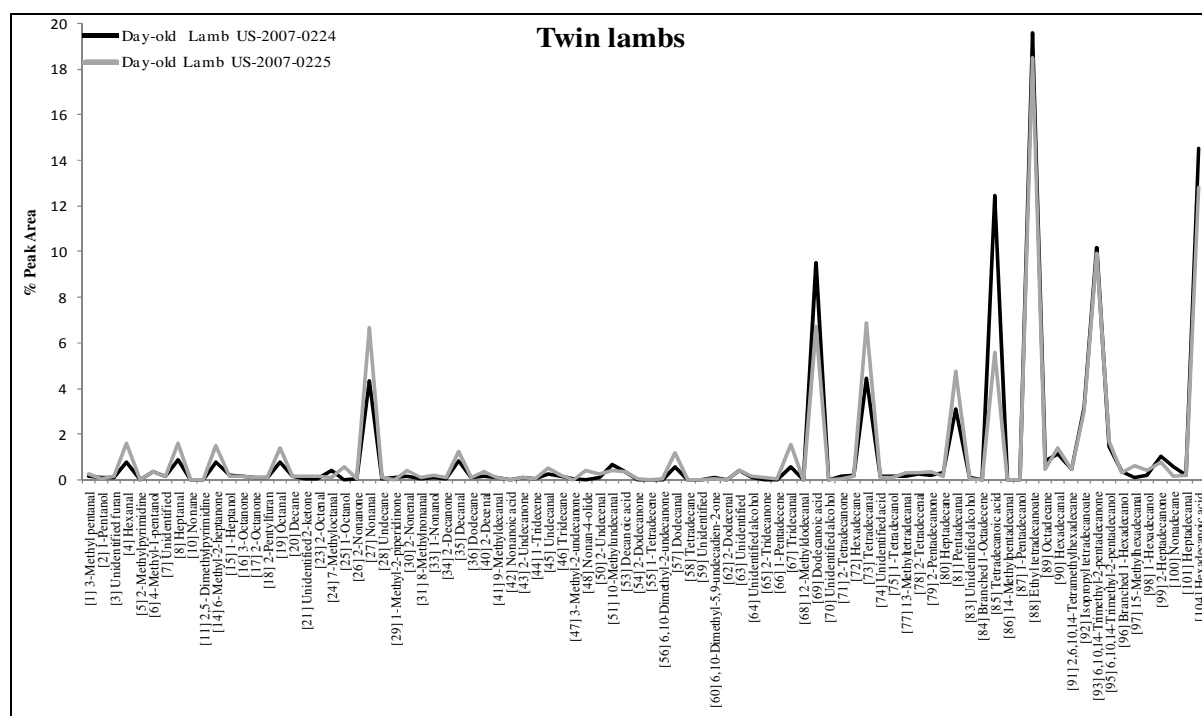


Fig. 4.9. Quantitative comparison (percentage peak area) of the wool volatiles of day-old twin lambs US-2007-0224 (black) and US-2007-0225 (grey).

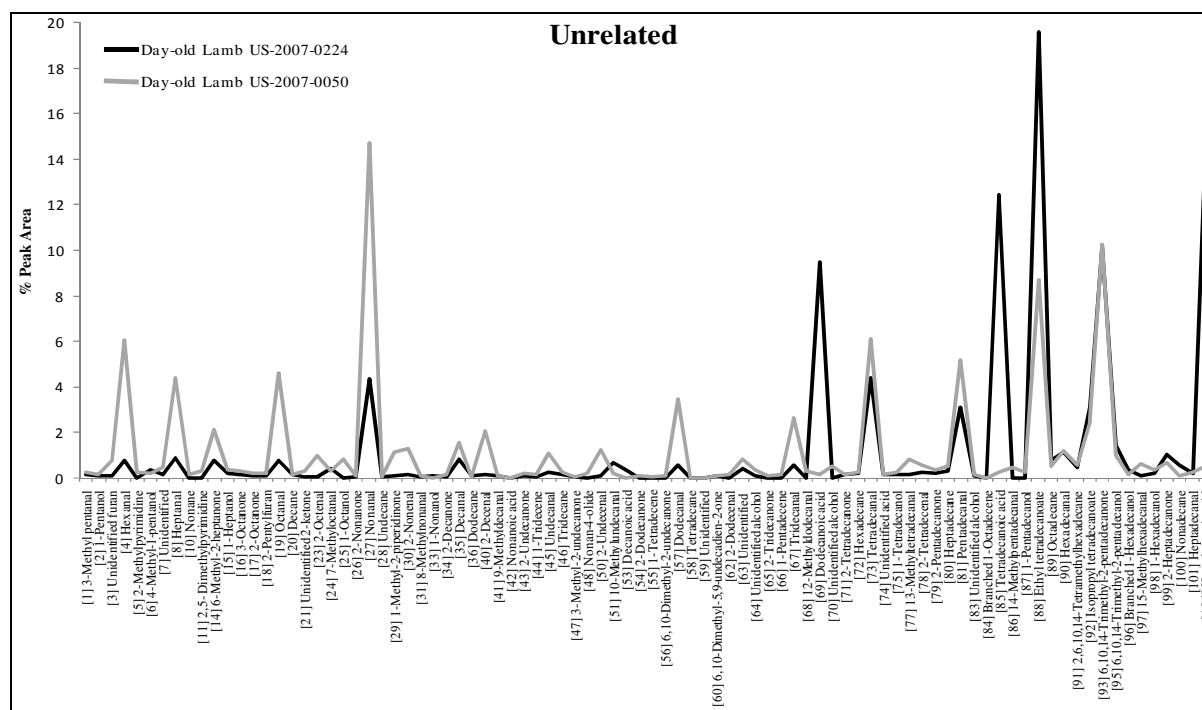


Fig. 4.10. Quantitative comparison (percentage peak area) of the wool volatiles of the unrelated lambs US-2007-0224 (black) and US-2007-0050 (grey).

#### 4.1.2.2 Wool from week-old lambs

Approximately 56% of the total quantity of volatile compounds present in the headspace of the wool of week-old Döhne Merino lambs born during the lambing season of 2007, taken as an average value for the 10 samples analysed, consists of only seven constituents: nonanal (**C27**) (24%), tetradecanal (**C73**) (8%), 6,10,14-trimethyl-2-pentadecanone (**C93**) (7%), heptanal (**C8**) (5%), dodecanoic acid (**C69**) (4%), octanal (**C19**) (4%) and dodecanal (**C57**) (4%) (Fig. 4.11). Interestingly, five out of these seven constituents are aldehydes.

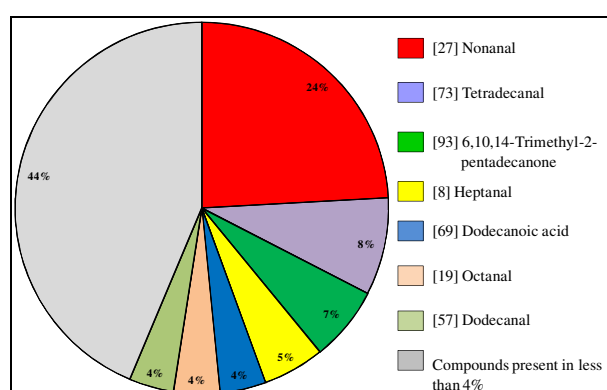


Fig. 4.11. Quantitative composition (percentage peak areas) of the wool volatiles collected from week-old Döhne Merino lambs during the lambing season of 2007.

Nonanal (**C27**) and 6,10,14-trimethyl-2-pentadecanone (**C93**) were found to be major constituents of the wool collected from day-old lambs in 2007 and 2009. Tetradecanal (**C73**) was another major constituent in wool samples collected in 2007.

A comparison of the TICs of the volatiles collected on day 1 and day 7 after birth from the wool of lamb US-2007-0516 reveals several obvious differences in the odour profiles of the two wool samples (Figs. 4.12 and 4.13).



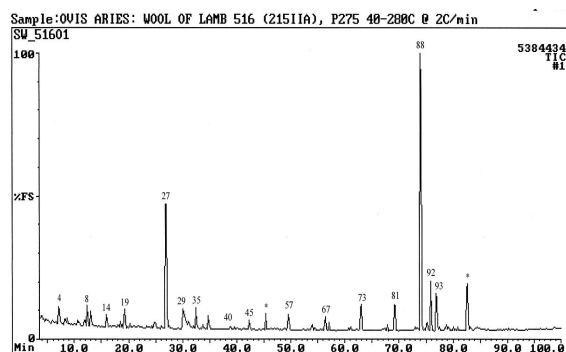


Fig. 4.12. TIC obtained by GC-MS analysis of the headspace of the wool of the day-old Döhne merino lamb US-2007-0516.

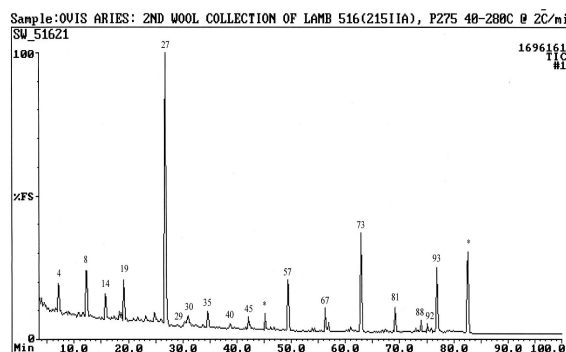


Fig. 4.13. TIC obtained by GC-MS analysis of the headspace of the wool of the week-old Döhne merino lamb US-2007-0516.

This can also be illustrated by superimposing the traces representing the percentage peak areas of the volatile constituents of wool collected from another lamb, US-2007-0224, on day-one and on day-seven after birth, as shown in Fig. 4.14.

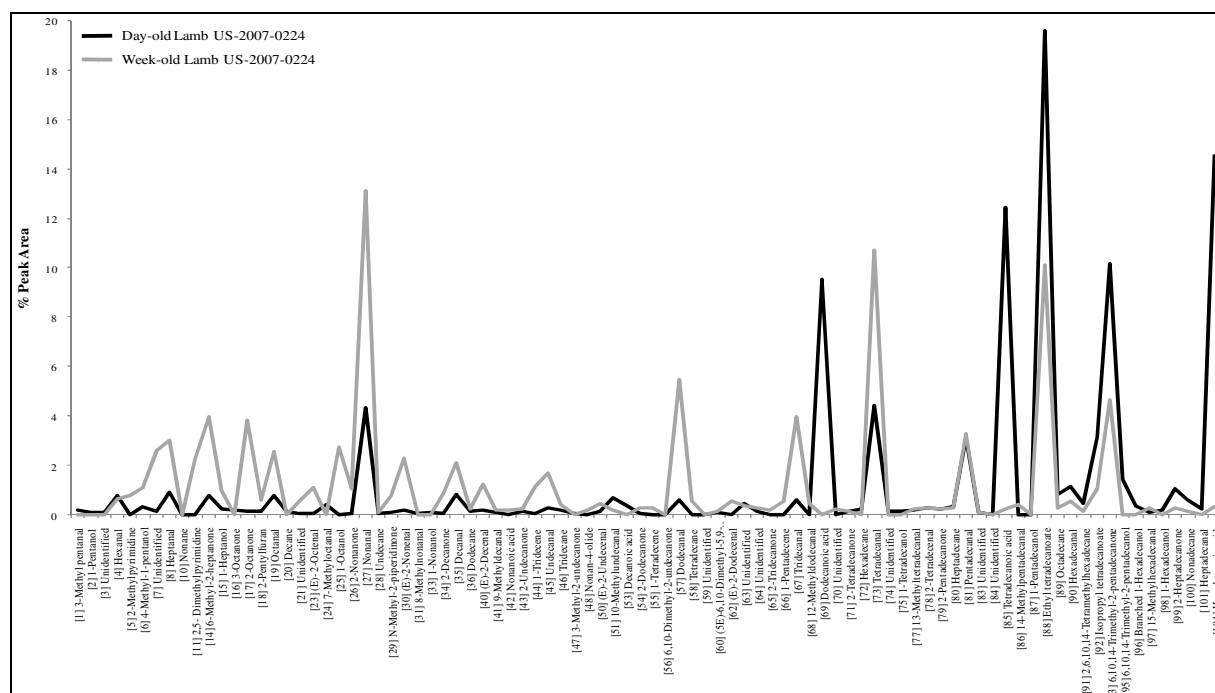


Fig. 4.14. Quantitative comparison (percentage peak area) of the wool volatiles of the lamb US-2007-0224 collected on day 1 (black) and day 7 (grey) after birth during the lambing season of 2007.

The changes in the average percentage peak areas of the wool volatiles collected during the lambing season of 2007 are shown in Figs. 4.15 to 4.16. In 51% of the constituents an increase in percentage peak area was observed, and 45% of the constituents showed an overall decrease in percentage peak area. 3-Methyl-2-undecanone (C47), 6,10-dimethyl-2-undecanone (C56), and the unidentified constituents C59 and C96 were only detected

---

---

in the wool collected on the first day after birth. In the light of the expected higher rate of evaporation of low-boiling compounds it is interesting that the more volatile compounds were mostly present in higher concentrations in the wool collected from week-old lambs, whereas the higher molecular mass compounds were present in higher concentrations in the wool collected from day-old lambs. An opinion regarding the basis of these changes can only be expressed once possibilities such as autoxidation of aldehydes to carboxylic acids, or of unsaturated long-chain compounds to shorter-chain aldehydes, the involvement of enzymes, etc., have been investigated.

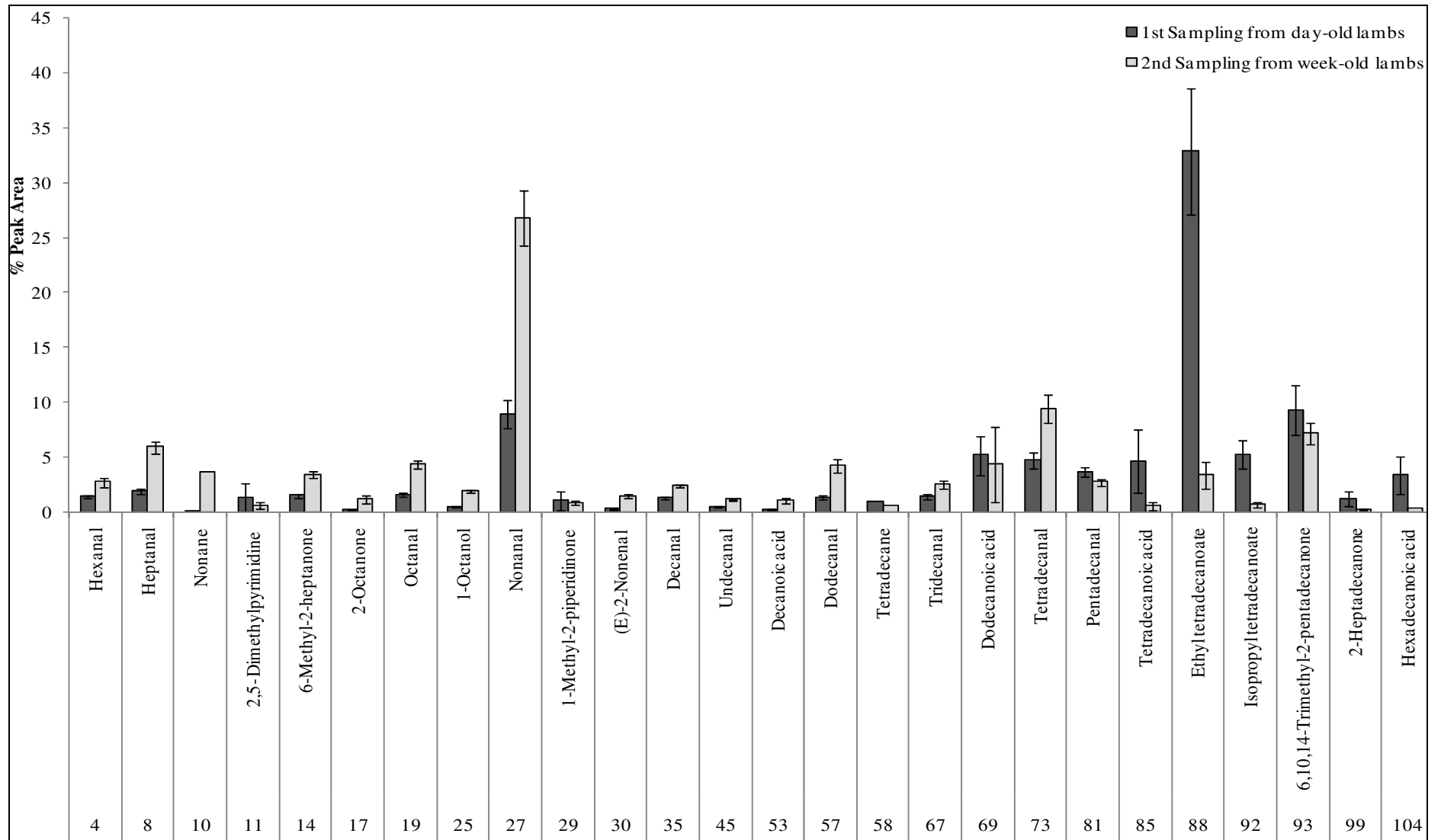


Fig. 4.15. Comparison of the percentage peak areas of the wool volatile present in percentages larger than 1% in the wool of day-old (black) and week-old lambs (grey) born during the lambing season of 2007. The constituents are numbered on the x-axis.

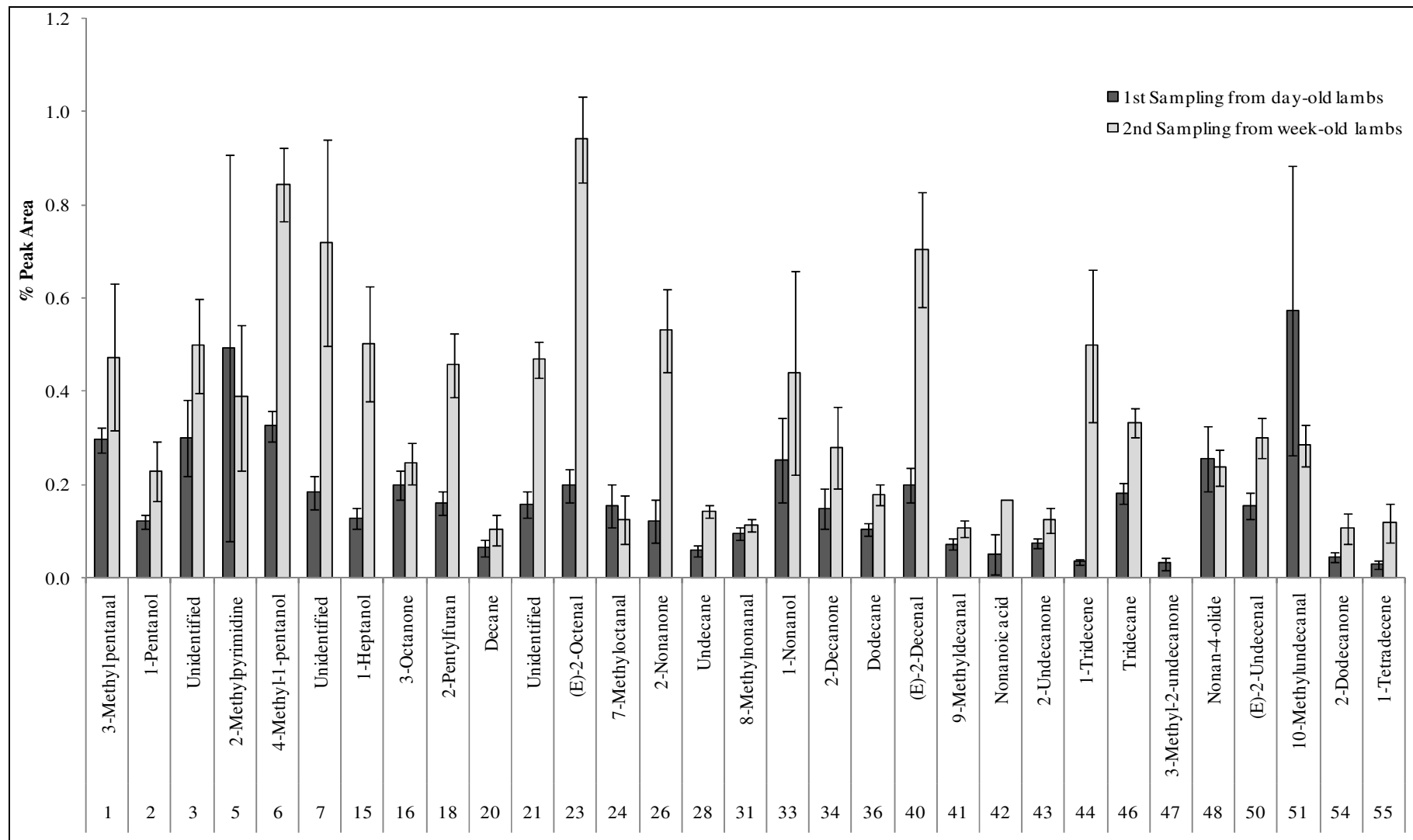


Fig. 4.16. Comparison of the percentage peak areas of the wool volatile present in percentages smaller than 1% in the wool of day-old (black) and week-old lambs (grey) born during the lambing season of 2007. The constituents are numbered on the x-axis. Part 1 of 2.

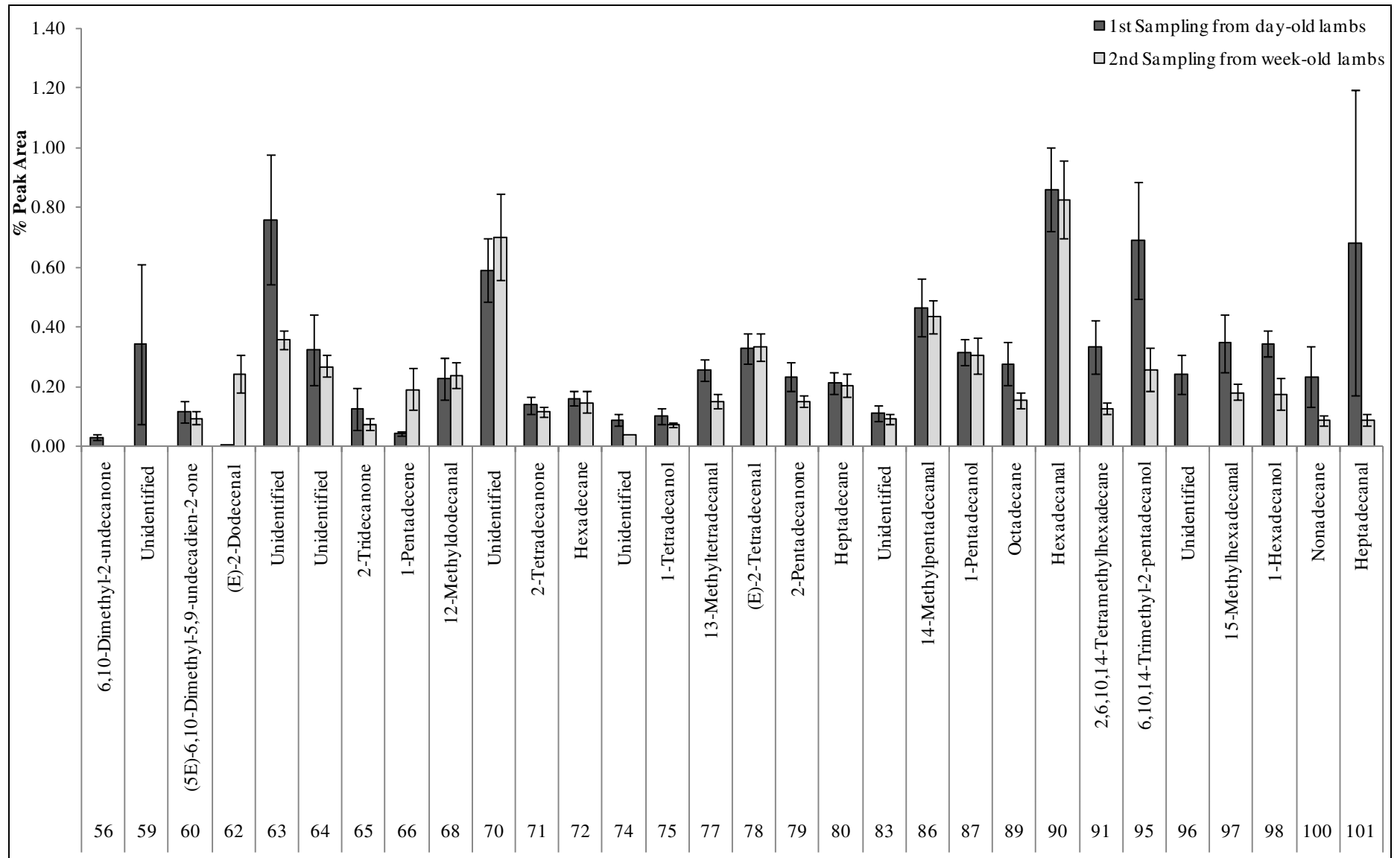


Fig. 4.16 Part 2 of 2.

The change in odour profile of the wool of a lamb born during the lambing season of 2009 was followed and the results were compared with the change in the odour profiles of the lambs that were born during the lambing season of 2007. Because of the high consumption of wool by the bioassays carried out in 2009, only one lamb (US-2009-0736) was available for this experiment, which was carried out towards the end of the lambing season. Small samples of cranial wool were collected from the lamb only on days one, five and seven because the lamb did not have enough wool for collection also on day 3, for example. Trends in the composition of the wool volatiles of this lamb are nevertheless apparent (Fig. 4.17).

A steady increase or decrease in the percentage peak area of 35% of constituents investigated was observed, whereas 56% of the constituents showed an overall increase and 38% of the constituents showed an overall decrease over the seven days. 3-Methylpentanal (**C1**), undecane (**C28**), 3-ethyl-4-methylpyrrolidine-2,5-dione (**C38**) and (*E*)-2-dodecenal (**C62**) were only identified in the wool collected on the first day after birth, and the two acids, nonanoic acid (**C42**) and decanoic acid (**C53**), were identified only in the wool samples collected a week after birth. The failure of some of the values measured on day 5 to follow the trend of the rest of the data cannot be explained until further experiments have been carried out.

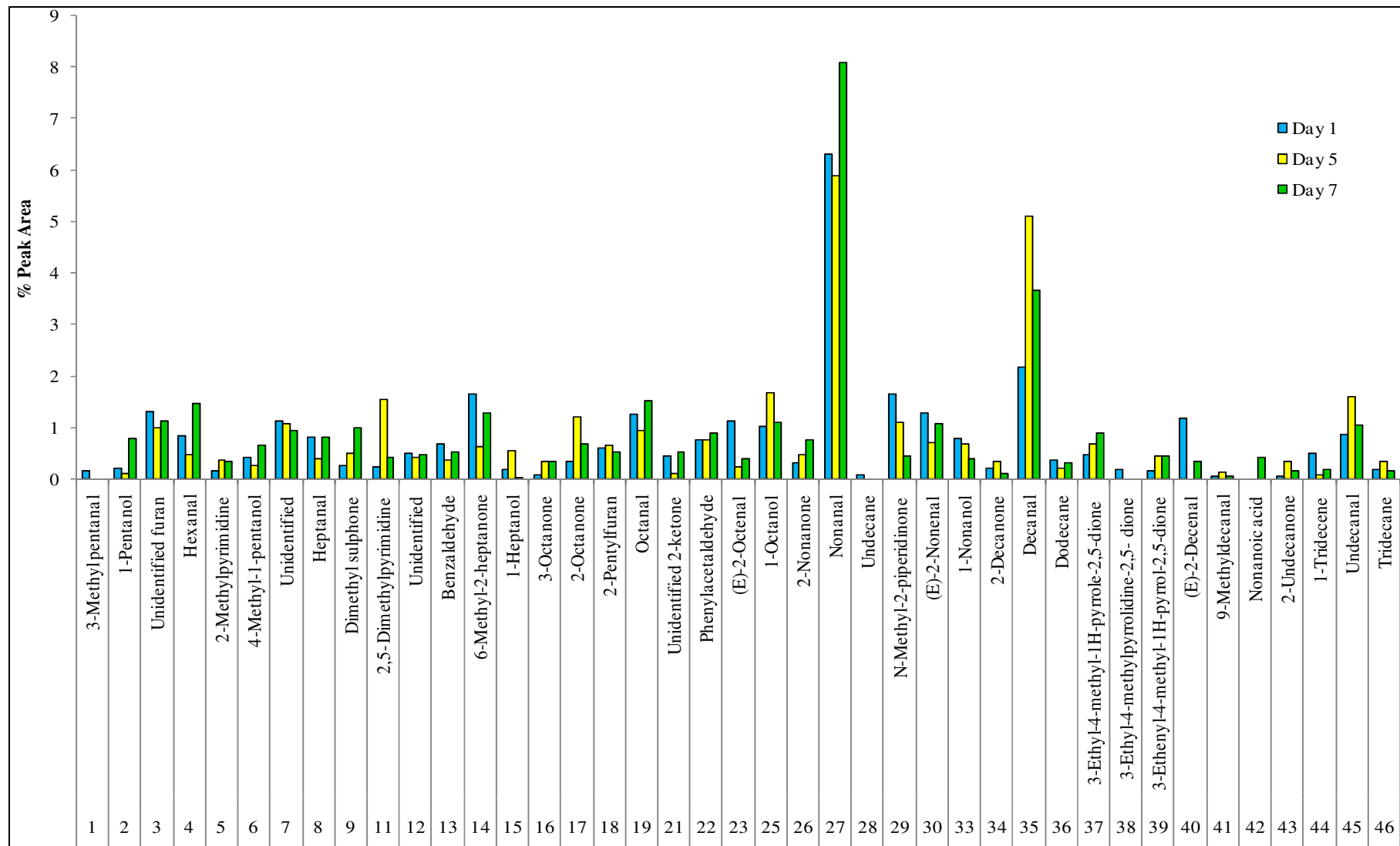


Fig. 4.17. Changes in percentage peak areas of the wool volatiles collected from lamb US-2009-0736 on day 1 (blue), day 5 (yellow) and day 7 (green) after birth during the lambing season of 2009. The constituents are numbered on the x-axis. Part 1 of 2.

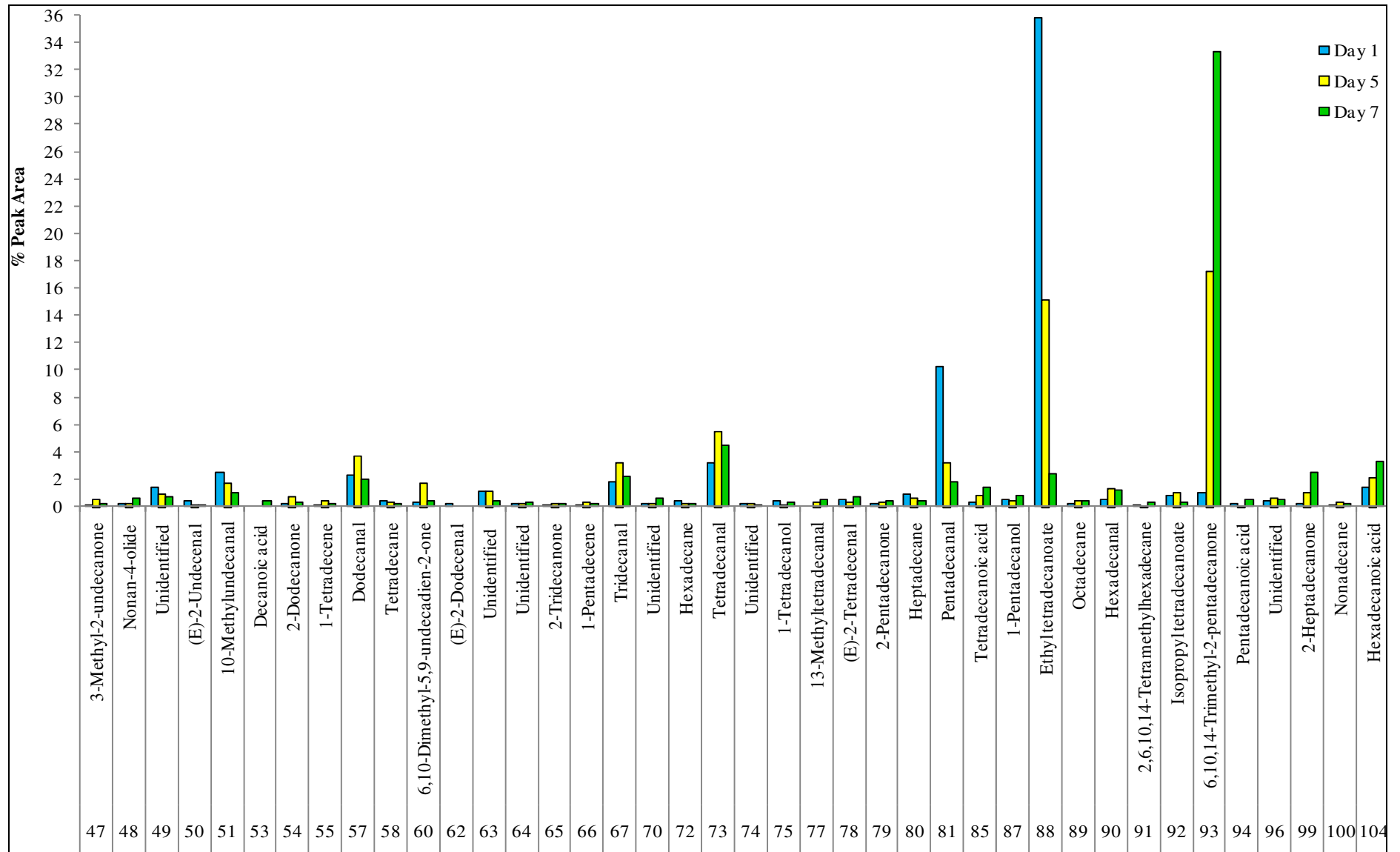


Fig. 4.17 Part 2 of 2.



Several wool volatiles that showed an increase or decrease in percentage peak area during the lambing season of 2007 also showed a corresponding increase or decrease in wool collected during the lambing season of 2009. The results are compared in Table 4.2.

**Table 4.2: Temporal changes in the percentage peak area of wool volatiles collected on days 1 and 7 after birth from lambs born during the lambing seasons of 2007 and 2009**

Higher on day 7		Lower on day 7	
Constituent		Constituent	
<b>2</b>	1-Pentanol	<b>29</b>	<i>N</i> -methyl-2-piperidinone
<b>4</b>	Hexanal	<b>53</b>	10-Methylundecanal
<b>6</b>	4-Methyl-1-pentanol	<b>58</b>	Tetradecane
<b>8</b>	Heptanal	<b>63</b>	Unidentified
<b>16</b>	3-Octanone	<b>72</b>	Hexadecane
<b>17</b>	2-Octanone	<b>74</b>	Unidentified
<b>19</b>	Octanal	<b>75</b>	1-Tetradecanol
<b>21</b>	Unidentified	<b>80</b>	Heptadecane
<b>25</b>	1-Octanol	<b>81</b>	Pentadecanal
<b>26</b>	2-Nonanone	<b>88</b>	Ethyl tetradecanoate
<b>27</b>	Nonanal	<b>92</b>	Isopropyl tetradecanoate
<b>35</b>	Decanal		
<b>43</b>	2-Undecanone		
<b>45</b>	Undecanal		
<b>55</b>	2-Dodecanone		
<b>56</b>	1-Tetradecene		
<b>66</b>	1-Pentadecene		
<b>67</b>	Tridecanal		
<b>70</b>	Unidentified		
<b>73</b>	Tetradecanal		
<b>78</b>	( <i>E</i> )-2-Tetradecenal		

Results obtained from the investigation of the change of the odour profile of lambs over the course of a week during the lambing season of 2007, as well as the investigation of the change of the odour profile of lamb US-2009-0736 during the lambing season of 2009, show that the quantitative composition of the odour of a lamb is not constant. The odours are continuously changing and as long as a ewe makes use of olfactory recognition as the main form of identification she has to continue learning the odour of the lamb.

#### 4.1.2.3 Wool from Döhne Merino ewes

Romeyer *et al.* (1993) showed that ewes use phenotype matching to recognise their lambs, since recognition was not influenced when the genetic make-up of the ewes differed from that of the lambs and neither was recognition influenced by the ewe's odour. First, this experiment (discussed in § 4.1.1) shows that a ewe does not compare her own odour to that of the lamb for recognition and, second, this shows that some elements of the lamb's odour could have a genetic origin (Wyatt, 2003: 109–110).

To gain insight into the possibility that maternal labelling could nevertheless have played a role in the outcome of the above experiment, wool was collected from the forehead of lamb US-2009-0736 and the qualitative and quantitative compositions of the lamb's wool volatiles were compared with the compositions of the volatiles of the wool collected from the forehead and the abdomen of this lamb's mother (Fig. 4.18). The wool was collected from the abdomen of the ewe because it could be argued that the volatiles present in the cranial wool of her lamb could have been picked up from the ewe when the lamb suckles or otherwise brings its head into contact with the ewe's abdomen. All compounds identified in the wool of the lamb were also identified in the wool of the ewe, except *N*-methyl-2-piperidinone (**C29**), 3-methyl-2-undecanone (**C47**) and 1-tetradecene (**C55**). This shows that the compounds used by the lamb are not unique, and that the same compounds are present in the wool of both ewe and lamb, although the concentrations of the compounds might differ, as it does in unrelated lambs. The age of the animal and its exposure to the environment might also influence its odour profile. With the exception of decanal (**C35**) and nonanoic acid (**C42**), no noteworthy differences were observed when the early-eluting constituents identified in the wool volatiles of the ewe were compared with those of the wool of the lamb (Fig. 4.18). The compounds with higher molecular masses, in particular 2-dodecanone (**C54**), ethyl tetradecanoate (**C88**) and 6,10,14-trimethyl-2-pentadecanone (**C93**) display greater differences in percentage peak areas.

In order to place the comparison of the odour profile of a ewe and her lamb in perspective, the odour profiles of three unrelated lambs are compared in Fig. 4.19. It is clear that the ewe and her lamb do not display odour profiles that are necessarily more similar than

---

---

the odour profile of the three randomly selected non-twin lambs. At least in the case of lamb US-2009-0736 maternal labels apparently did not play a major role in the pheromone communication between the ewe and the lamb. It also has to be taken into consideration that the odour profile of the wool of the ewe can be expected to differ from the odour profile of the lamb, since the wool of the ewe was exposed to environmental contaminants for a longer period than the wool of the lamb.

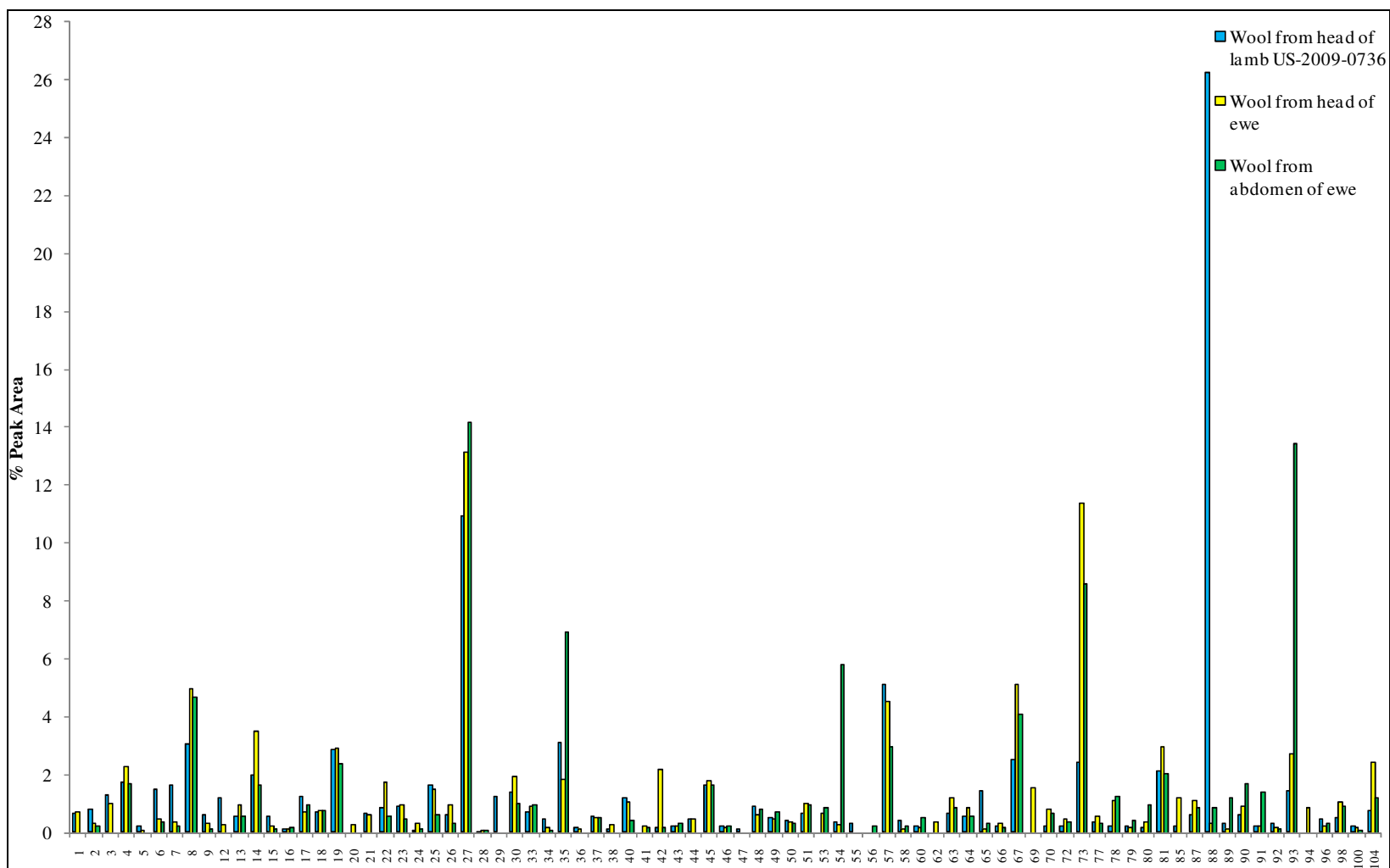


Fig. 4.18. Comparison of the percentage peak areas of the wool volatiles collected from the head of lamb US-2009-0736 (blue), and the wool collected from the head (yellow) and the abdomen (green) of this lamb's mother during the lambing season of 2009. Compound numbers are indicated on the x-axis of the graph.

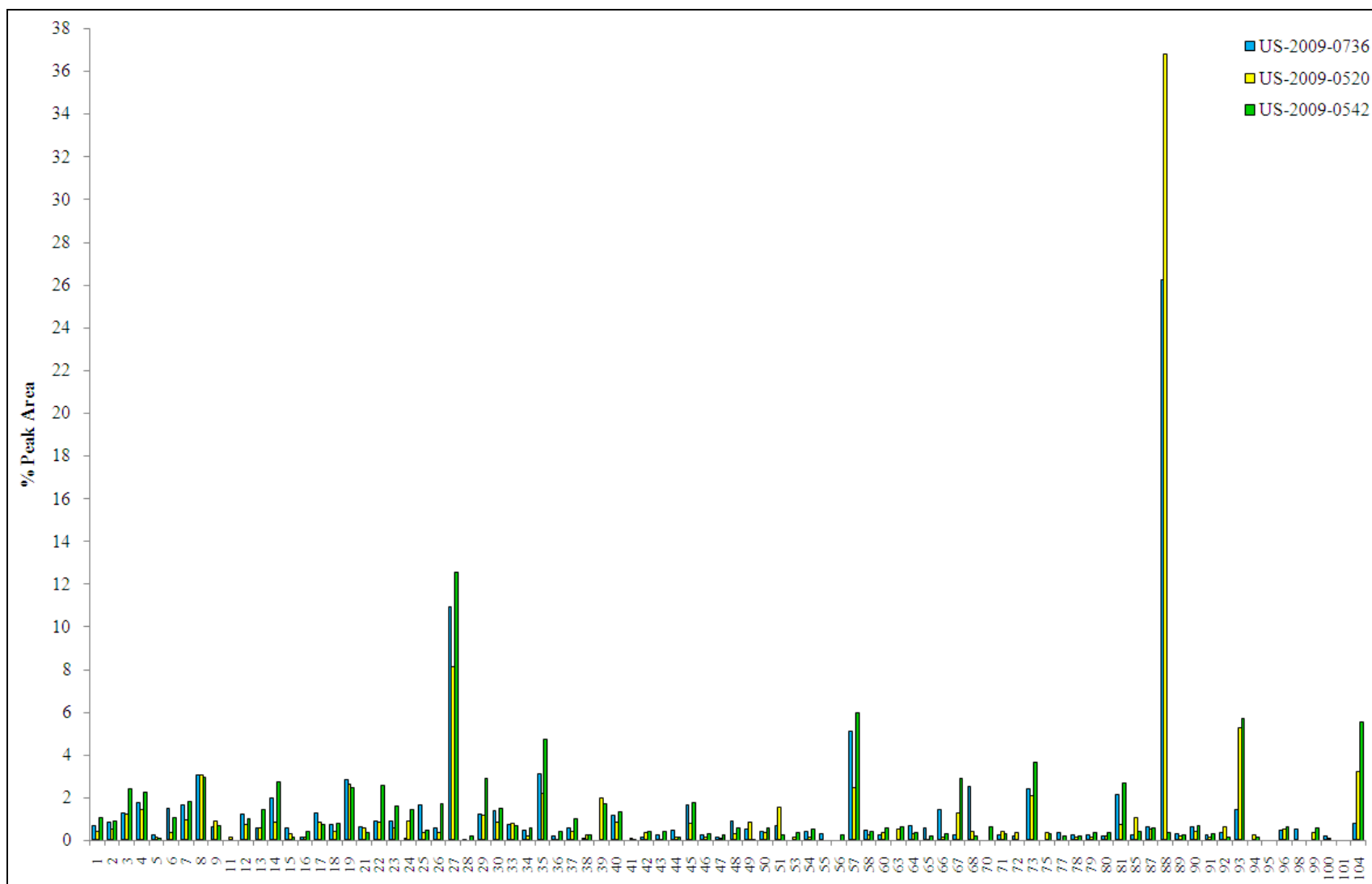


Fig. 4.19. Comparison of the percentage peak areas of the wool volatiles collected from the heads of the unrelated lambs US-2009-0736 (blue), US-2009-0520 (yellow) and US-2009-0548 (green) born during the lambing season of 2009. Compound numbers are indicated on the x-axis of the graph.

### 4.1.3 Statistical analysis

Studying semiochemicals involved in kin recognition poses a significant problem for analytical chemists. Samples of secretion, excretion or effluvium of the organism under investigation may contain a multitude of compounds detectable by chromatography, but the constituents of the pheromone might not be the compounds that are present in the highest concentrations in the collected material. This has to be kept in mind when using statistical analysis since those constituents identified as principal components using multivariate analyses might not necessarily be the constituents the organism considers to be principal components. Nevertheless, the vast amount of data generated in the investigation into the composition of volatile constituents present in the wool of the lambs made multivariable methods of analysis indispensable in attempts to reach a meaningful interpretation of the available data. The information on the lambs that were used as a sample group is summarised in Table 4.1.

Multivariate analyses were used to further investigate the different aspects of kin recognition discussed earlier. Firstly, the genetic influence on the odour profile of the lambs was investigated, *i.e.*, whether twin lambs possess odour profiles that are more similar than are those of any two other randomly selected non-twin lambs. Furthermore, the wool collected from lambs born of the same ewes in 2007 and 2009 were analysed to establish whether lambs born of a specific ewe, even years apart, display odour profiles that are more similar than are those of lambs born of different ewes. Secondly, the change in the odour of the lambs over a one-week period was assessed to determine whether the odour changes in a predictable and uniform manner. The odour profiles of a twin were once again evaluated for their similarity and compared with those of two randomly selected non-twin lambs to determine whether the intra-twin similarities and inter-twin differences are retained over a period of one week.

#### 4.1.3.1 Genetic influence on the odour profiles of twin lambs

The results of the quantitative analyses of the wool volatiles collected from 32 day-

old lambs born of 16 ewes, representing 9.69% of the ewe flock during the lambing season of 2007, were used for statistical investigation. Of the 133 constituents discussed in Chapter 3, only those present in 20% or more of the wools samples were considered as variables for statistical analysis. This reduced the number of constituents to 88. The chromatographic data used for multivariate analyses were characterised by a large number of variables (*i.e.* the peak areas for all the identified constituents,  $n \leq 88$ ) relative to the number of sample units (wool samples,  $n \leq 32$ ). Peak areas were normalised across all samples to produce variables that can be compared with one another (Kowalski and Bender, 1972).

Permutation tests were carried out to determine whether the wool of twin lambs can be grouped according to their qualitative and quantitative composition of the volatile organic material present in their wool. This testing procedure involves of the following: first, an intra-twin and inter-twin sum of squares were calculated to obtain an *F*-statistic as in ordinary one-way analysis of variance. Then the lambs were randomly divided into 16 pairs of lambs and the *F*-statistic computed as it was calculated for the original twins. This step was repeated 10 000 times to form an approximation of the full permutation distribution. The achieved significance level (*P*-value) was calculated by computing the proportion of times the permutation *F*-values exceeded the actual *F*-value obtained for real twin lambs. A *P*-value is related to a null hypothesis test; it is the probability of obtaining a result as extreme or more extreme than the one observed if the null hypothesis is true (Dawson-Saunders and Trapp, 1994: 93). In this study, the null hypothesis was that groups based on the criteria of being twins do not differ in the qualitative and quantitative composition of their wool volatiles from other twin groups. Thus, the calculated *P*-value provides an indication of whether the differences found between the twin lambs can be attributed to the fact that they are twins or to random variation in the data. *P*-values range from 0 to 1 and when a *P*-value is less than 0.05 percent, the null hypothesis is rejected (Gower, 1999). This would indicate that the twins form statistically significant different groups. Fig. 4.20 explains the significance of the *P*-value in the present study. The grey bars in the histograms in the lower part of the figure represent the permutation distribution and the red star represents the position of the calculated *P*-value. The calculated *P*-value is the proportion of the area under the histogram. If there is significant similarity in the composition of the wool

of twin lambs compared to the composition of the wool of other lambs then the observed distances between the  $P$ -value calculated for the twins will be larger than the distances resulting from the permutation tests of randomly paired non-twin lambs. In other words, the position of the star in the figure (that represents the  $P$ -value calculated for twins) will fall into the tail area of the histogram. If the odour profiles of the twins is as similar as that of any other randomly paired non-twin lambs, the distance between the  $P$ -value calculated for twin lambs and that of the randomly paired data will be the same, and the star will be positioned close to the centre of the histogram. Thus, the further the star is positioned from the centre of the histogram, the greater the uniqueness of the odour profile of twins will be compared to that of randomly paired non-twin lambs.

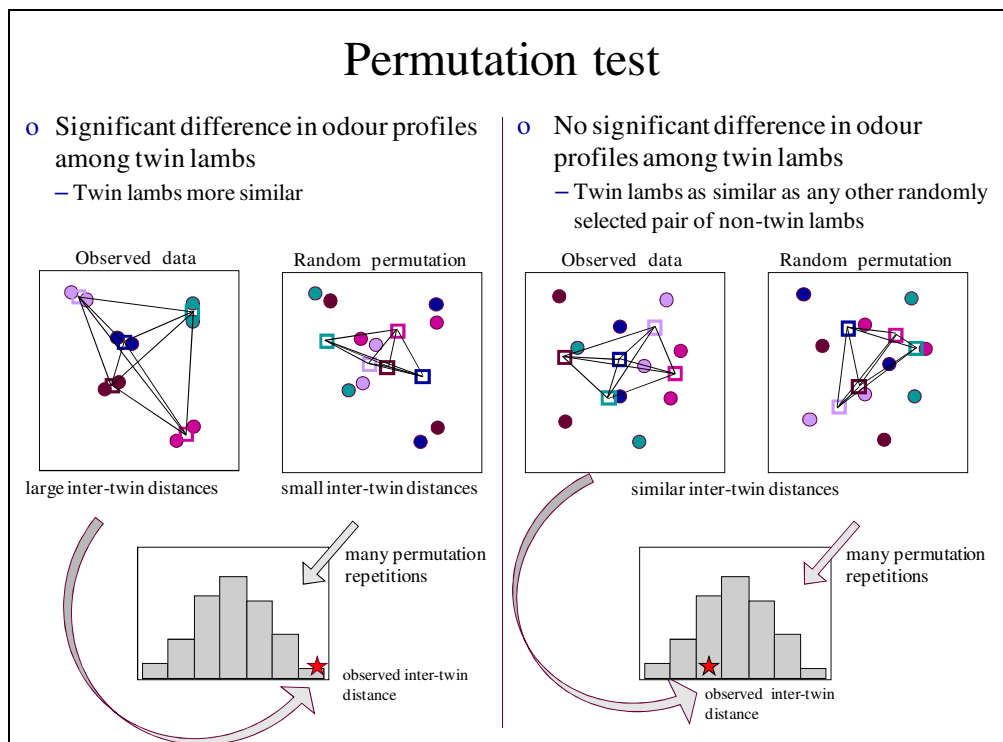


Fig 4.20. Schematic representation of how the  $P$ -value was obtained (Le Roux and Lubbe, 2009).

The following nul-hypothesis was tested: groups based on the criteria of twins do not differ in the qualitative and quantitative composition of their wool. Using this principle, the similarity in the odour profile of the wool of a pair of twin lambs was assessed by



executing 10 000 permutation tests. A  $P$ -value of 0.0000 (*i.e.* approximately zero) was calculated, indicating that the pairing of the twin pairs of lambs according to the qualitative and quantitative composition of the wool did not occur by chance, and is statistically significant. The permutation distribution in the form of a histogram is shown in Fig. 4.21 and the calculated  $P$ -value of 0.000 is indicated by the red line on the right hand side of the figure. The  $P$ -value is the proportion of the area under the histogram, in this case it is zero and the null hypothesis is thus rejected. Twin lambs thus possess odour profiles that are unique and their odour profiles are more similar than those of other randomly selected non-twin lambs.

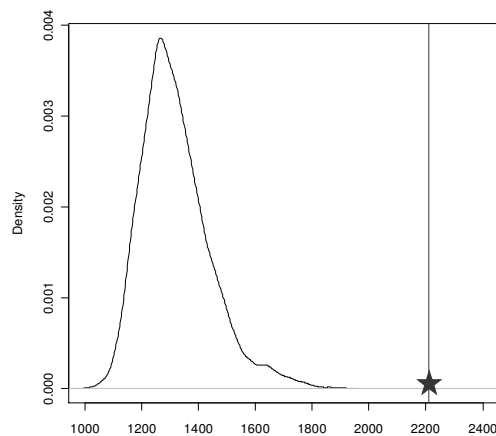


Fig 4.21. Permutation distribution showing the differences in the composition of the wool volatiles collected from day-old twin lambs compared to the rest of the group of lambs.

The 88 constituents, and the quantities in which they are found in the wool of day-old lambs born during the lambing season of 2007, were used to construct a PCA biplot in order to obtain insight into the multivariate character of the results (Figs. 4.22 and 4.23). Biplots can be considered as multivariate scatterplots that simultaneously give a graphical presentation of samples (lambs) and variables (identified constituents). Lambs are displayed as points on the graph while the identified constituents are displayed as linear axes. Each axis of the biplot is used similar to the usage of an axis in an ordinary scatterplot: a line is drawn from any point in a biplot perpendicular to a biplot axis and the value of the point for

---

---

that variable is read from the axis. In addition to this usage of biplot axes, the angles between any two axes is an approximation of the correlation between the relevant two variables. Constituents displayed as axes that are 90 degrees in relation to one another have no correlation with each other, and those constituents lying on axes close to one another have a high level of correlation with one another. An axis is labelled at the positive value of its calibration. In this study, a PCA biplot provides the optimal two-dimensional presentation of the data matrix. Not all 32 samples and 88 variables are equally well represented in the biplot and axis predictivity (Gardner-Lubbe *et al.*, 2008). The quality of a biplot is an overall measure of the accuracy of the two-dimensional approximation of the data matrix. Furthermore, sample predictivities and axis predictivities provide detailed information about the how accurately each data point is represented in the biplot and the degree of accuracy in the predictions made from a biplot axis (Gardner-Lubbe *et al.*, 2008). The predictivity values range from 0 to 1, with a value of 1 representing the best predictivity. In Fig. 4.22 only the 21 axes with predictivities higher than 0.800 are displayed in the PCA biplot. The quality of the display in the PCA biplot was measured and this value reflects the proportion of the variation in the data accounted for in the two-dimensional display (Gower and Hand, 1996). The quality of display for the PCA biplots in Figs. 4.22 and 4.23 is 54%. This means that 54% of the variation of the data is explained in the first two dimensions of the two-dimensional biplot; the other 46% is explained in the remaining 30 dimensions. In Fig. 4.23 the axes are not displayed in order to draw attention to the positions of the twin lambs. Each square represents a sample of wool collected from a day-old lamb, and the last three digits of the lamb's number (as given in Table 4.1) are indicated on the biplot. The lambs of each of the 16 twins are connected with black lines in Figs. 4.22 and 4.23.

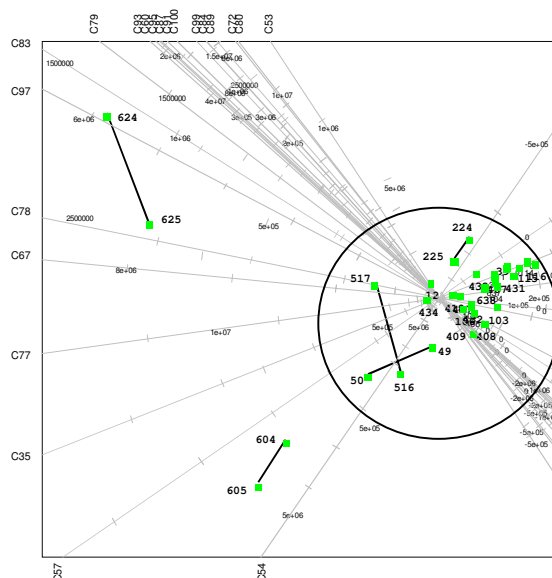


Fig. 4.22. PCA biplot for the wool volatiles collected from day-old lambs during the lambing season of 2007.

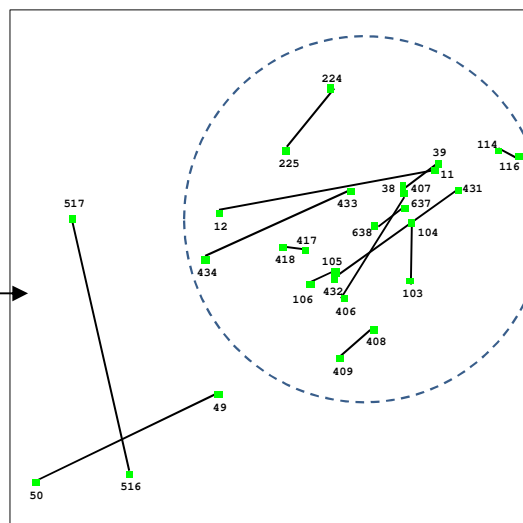


Fig. 4.23. PCA biplot displaying the crowded area in Fig. 4.22. Lambs possessing similar odour profiles are encircled.

The group of lambs concentrated in the area encircled in the biplot in Fig. 4.23 indicates that they possess odour profiles that are generally similar.

The 21 constituents with predictivities higher than 0.800 in order of decreasing predictivity are 2,6,10,14-tetramethylhexadecane (**C91**), 2-pentadecanone (**C79**), octadecane (**C89**), 1-pentadecanol (**C87**), (5*E*)-6,10-dimethyl-5,9-undecadien-2-one (**C60**), 15-methylhexadecanal (**C97**), unidentified constituent **C84**, (*E*)-2-tetradecenal (**C78**), 2-dodecanone (**C54**), tridecanal (**C67**), decanoic acid (**C53**), decanal (**C35**), 6,10,14-trimethyl-2-pentadecanol (**C95**), 13-methyltetradecanal (**C77**), hexadecane (**C72**), heptadecane (**C80**), nonadecane (**C100**), 2-heptadecanone (**C99**), unidentified constituent **C83**, 6,10,14-trimethyl-2-pentadecanone (**C93**) and dodecanal (**C57**). Interestingly, all the aldehydes have their maxima on the right hand side of the plot; dodecanal is situated on the bottom right hand side of the plot. All the methyl ketones, except 2-dodecanone, and all the hydrocarbons and decanoic acid, are highly correlated with one another.

Wool samples collected in 2009 from eight day-old lambs of five of the ewes that belonged to the sample group in 2007 were used for a further statistical investigation. As in the case of the wool volatiles collected in 2007, only the volatiles present in 20% or more of the samples collected in 2009 were considered as variables for statistical analysis, which reduced the variables to 84 constituents. The similarity in the odour profiles of the wool of a pair of twin lambs was assessed by executing 10 000 permutation tests. A *P*-value of 0.0064 was calculated, indicating that the null hypothesis is rejected, and that the investigated twin lambs born during the lambing season of 2009 thus possess odour profiles that are unique and their odour profiles are more similar than those of any other randomly selected non-twin lambs. A higher *P*-value was obtained for the lambs investigated in 2009, compared to those investigated in 2007, but this can most likely be attributed to the smaller number of wool samples that were available in 2009; wool samples of only eight lambs were available in 2009, compared to the 32 lambs investigated in 2007.

A PCA biplot was used to gain insight into the multivariate character of the qualitative and quantitative composition of the wool volatiles of the lambs in 2009. Twins are again connected by lines in Fig. 4.24. The quality of display of the PCA biplot is 56% and only the 27 constituents with predictivities higher than 0.800 are displayed in the biplot (Fig. 4.24). These constituents, in order of decreasing predictivity, are nonanal (**C27**), hexanal (**C4**), 7-methyloctanal (**C24**), heptanal (**C8**), nonan-4-olide (**C48**), 2-tetradecanone (**C71**), (*E*)-2-nonenal (**C30**), octanal (**C19**), 6,10,14-trimethyl-2-pentadecanol (**C95**), benzaldehyde (**C13**), 3-octanone (**C16**), octadecane (**C89**), 2-heptadecanone (**C99**), 2-decanone (**C34**), 2-octanone (**C17**), (*E*)-2-decenal (**C40**), tetradecane (**C58**), 6,10,14-trimethyl-2-pentadecanone (**C93**), 3-ethyl-4-methyl-1H-pyrrole-2,5-dione (**C37**), 1-octanol (**C25**), 6,10-dimethyl-2-undecanone (**C56**), unidentified constituent **C3**, 3-methyl-2-undecanone (**C47**), 2-methylpyrimidine (**C5**), 2,6,10,14-tetramethylhexadecane (**C91**), nonadecane (**C100**) and 2-pentadecanone (**C79**).

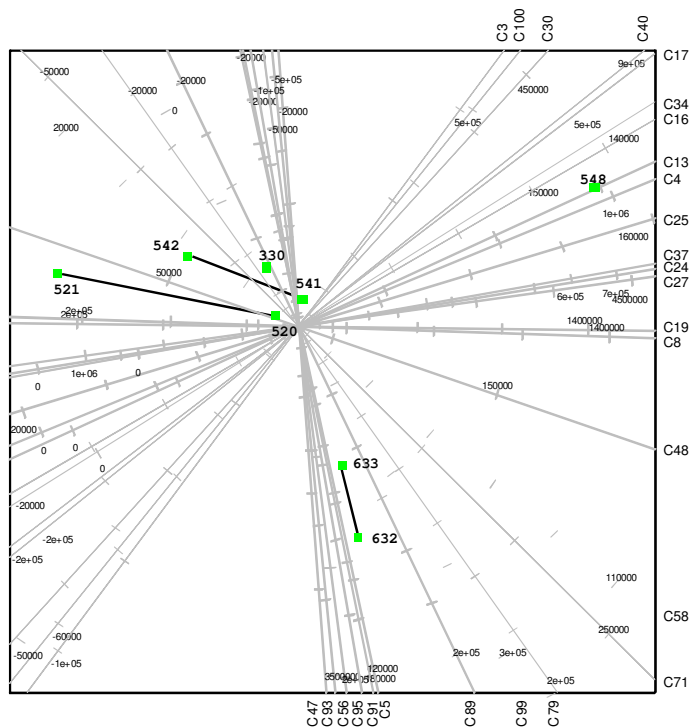


Fig. 4.24. PCA biplot for the wool volatiles collected from day-old lambs during the lambing season of 2009.

Constituents 2-pentadecanone (**C79**), octadecane (**C89**), 6,10,14-trimethyl-2-pentadecanone (**C93**), 2-heptadecanone (**C99**), 2,6,10,14-tetramethyl-hexadecane (**C91**) and nonadecane (**C100**) are among the constituents with high predictivity in the wool volatiles collected in 2007 as well as in 2009.

The wool volatiles collected from lambs born during 2007 and 2009 of the same ewes were compared to determine whether the lambs born of the same ewe two years later possess odour profiles that are more similar than those of other randomly selected non-twin lambs. In Figs. 4.25 and 4.26 the TICs of the odour profiles of wool samples from lamb US-2007-0103 and lamb US-2009-0330, both born of ewe US-2002-0424, in 2007 and 2009, respectively, are compared.

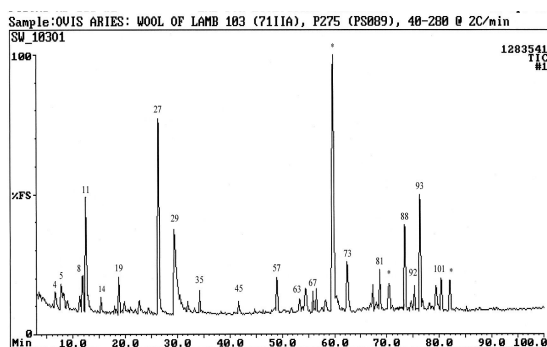


Fig. 4.25. TIC of the SEP enriched wool volatiles of the day-old lamb US-2007-0103 of ewe US-2002-0424.

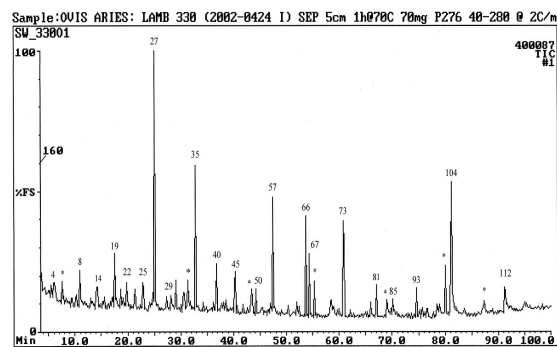


Fig. 4.26. TIC of the SEP enriched wool volatiles of the day-old lamb US-2009-0330 of ewe US-2002-0424.

The odour profiles were not significantly different, since nearly all the constituents identified in 2007 were also present in the wool collected in 2009. However, the PCA biplot in Fig. 4.27 shows a definite separation between the wool volatiles from lambs born during 2007 and those from lambs born during 2009, indicating that there are substantial quantitative differences between the volatiles of the two groups of lambs. This provides strong evidence that the odour profiles of lambs born of the same ewe in different lambing seasons are not identical within the limits of reproducibility of the analytical method that was used.

The quality of display of the PCA biplot in Fig. 4.27 is 56% and only the 20 constituents with predictivities higher than 0.800 are displayed in the biplot (Fig. 4.27). The constituents, in order of decreasing predictivity, are tridecane (**C46**), 13-methyltetradecanal (**C77**), 3-octanone (**C16**), 1-heptanol (**C15**), hexanal (**C4**), unidentified constituent **C64**, tridecanal (**C67**), (*E*)-2-tetradecenal (**C78**), octanal (**C19**), (*E*)-2-nonenal (**C30**), 2-pentadecanone (**C79**), tetradecanal (**C73**), dodecanal (**C57**), hexadecane (**C72**), 6-methyl-2-heptanone (**C14**), (*E*)-2-octenal (**C23**), heptanal (**C8**), *N*-methyl-2-piperidinone (**C29**), hexadecanal (**C90**) and 2,5-dimethylpyrimidine (**C11**). It is clear from Fig. 4.27 that the lambs born during 2007 contain higher amounts of all the above-mentioned constituents. The two nitrogen containing constituents, *N*-methyl-2-piperidinone (**C29**) and 2,5-dimethylpyrimidine (**C11**), are highly correlated with one another. All the hydrocarbons, ketones and the aldehydes, except (*E*)-2-octenal (**C23**), are also reasonably well correlated with each other.

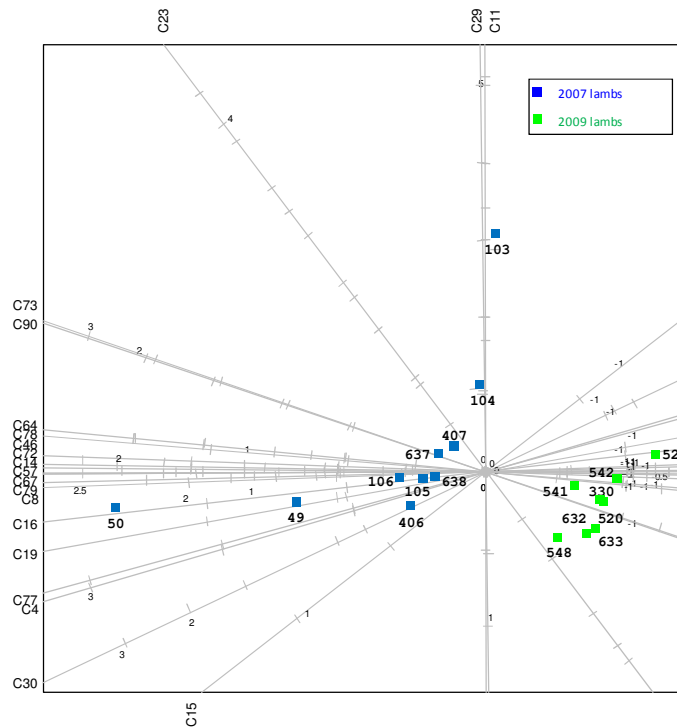


Fig. 4.27. PCA biplot for the wool volatiles collected from day-old lambs during the lambing seasons of 2007 and 2009.

#### 4.1.3.2 Change in the lamb odour profiles over a one-week period

Wool samples collected from five week-old twins (10 lambs) born of five ewes during the lambing season of 2007 were used for statistical investigation. The 88 constituents that were used in statistical analysis of the wool volatiles of day-old lambs were used as variables in statistical analysis of this dataset. The similarity in the odour profiles of the wool of twins was assessed by executing 10 000 permutation tests. A *P*-value of 0.0099 was calculated, indicating that the pairing of the twin lambs according to the quantitative composition of their wool volatiles is statistically significant. The somewhat higher *P*-value obtained for week-old lambs compared to day-old lambs could be ascribed to the smaller sample size of only 10 lambs, as discussed above in the previous case of the smaller sample size of the day-old lambs born during 2009.

The 88 constituents, and the quantities in which they are present in the wool of the lambs, were used to construct a PCA biplot in order to obtain insight into the multivariate

character of the results (Fig. 4.28). The quality of display for the PCA biplot in Fig. 4.28 is 64% and only the 24 constituents with predictivities higher than 0.800 are displayed in the PCA biplot. Each green square represents a sample of wool collected from a week-old lamb and the twins are connected with black lines.

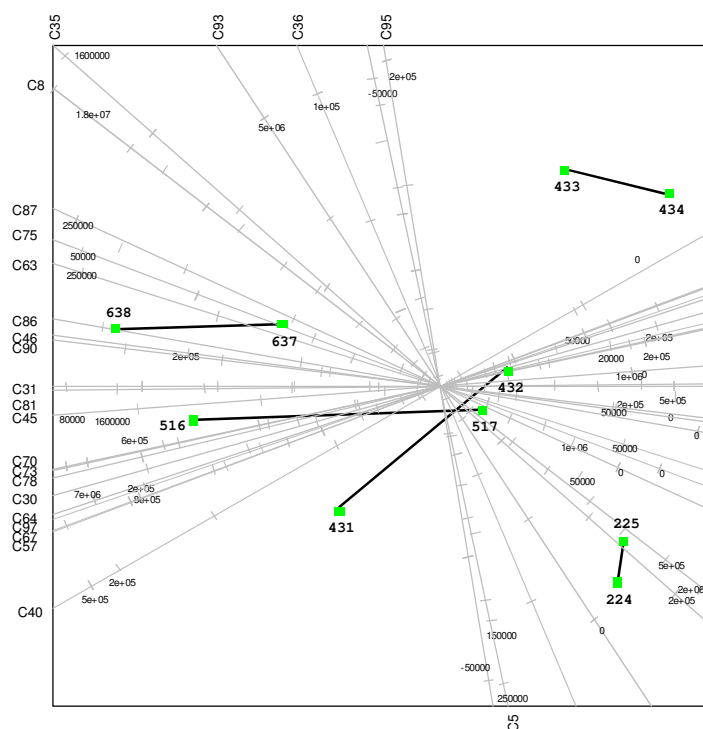


Fig. 4.28. PCA biplot for the wool volatiles collected from week-old lambs during the lambing season of 2007.

The constituents that best represent the predictivity in the quantitative composition of the wool of week-old lambs, in order of decreasing predictivity, are decanal (**C35**), dodecanal (**C57**), unidentified constituent **C64**, tridecanal (**C67**), tetradecanal (**C73**), heptanal (**C8**), unidentified constituent **C63**, 8-methylnonanal (**C31**), tridecane (**C46**), pentadecanal (**C81**), (*E*)-2-tetradecenal (**C78**), undecanal (**C45**), 6,10,14-trimethyl-2-pentadecanol (**C95**), 6,10,14-trimethyl-2-pentadecanone (**C93**), hexadecanal (**C90**), 15-methyl-hexadecanal (**C97**), dodecane (**C36**), 2-methylpyrimidine (**C5**), (*E*)-2-nonenal (**30**), unidentified constituent **C70**, 1-pentadecanol (**C87**), (*E*)-2-decenal (**C40**), 1-tetradecanol (**C75**) and 14-methylpentadecanal (**C86**).



When considering Fig. 4.28, it is clear that even though the odour profiles of the lambs change over a seven-day period (as explained in § 4.1.2.2), the similarity in odour profiles within the pairs of twin lambs is not lost, since the twins are still grouped reasonably closely to one another. This becomes even more apparent when the quantitative composition of the wool of day-old lambs is compared to that of week-old lambs in Fig. 4.29. The separation of the day-old wool samples from the week-old samples indicates that a complete change in the odour profile of the wool has taken place during the seven days, and, since no overlapping of the wool samples collected from day-old and week-old lambs is observed, a uniform change in the odour profile of all the lambs has apparently taken place. The similarities in the odour profiles of the twin lambs are not lost, since the individuals of the pairs of twin lambs are still grouped together. The distances between the twin lambs decrease from day-old lambs to week-old lambs, indicating that the odours of twins are more similar a week after birth than on the first day after birth. All the wool samples collected from the lambs are grouped closely together, further indicating that the odours of week-old lambs are generally more similar than the odour of day-old lambs. The quality of display of the PCA biplots in Fig. 4.29 is 58%, and only the nine constituents with predictivities higher than 0.800 are displayed on the plot. These constituents, in order of decreasing predictivities, are 8-methylnonanal (**C31**), undecanal (**C45**), decanal (**C35**), 1-hexadecanol (**C98**), hexadecanal (**C90**), heptadecane (**C80**), 2-tetradecanone (**C71**), 1-tetradecanol (**C75**) and 2-pentadecanone (**C79**). According to the biplot shown in Fig. 4.29, the higher molecular weight constituents, **C98**, **C90**, **C80**, **C71**, **C75** and **C79**, are highly correlated with one another, and the wool collected from day-old lambs contains higher concentrations of these constituents than the wool from week-old lambs. The more volatile, lower molecular weight constituents **C31**, **C45** and **C35** also display correlation with one another, and all the lambs, except lambs US-2007-637 and US-2007-638, contain higher amounts of these constituents in their wool a day after birth than a week later.

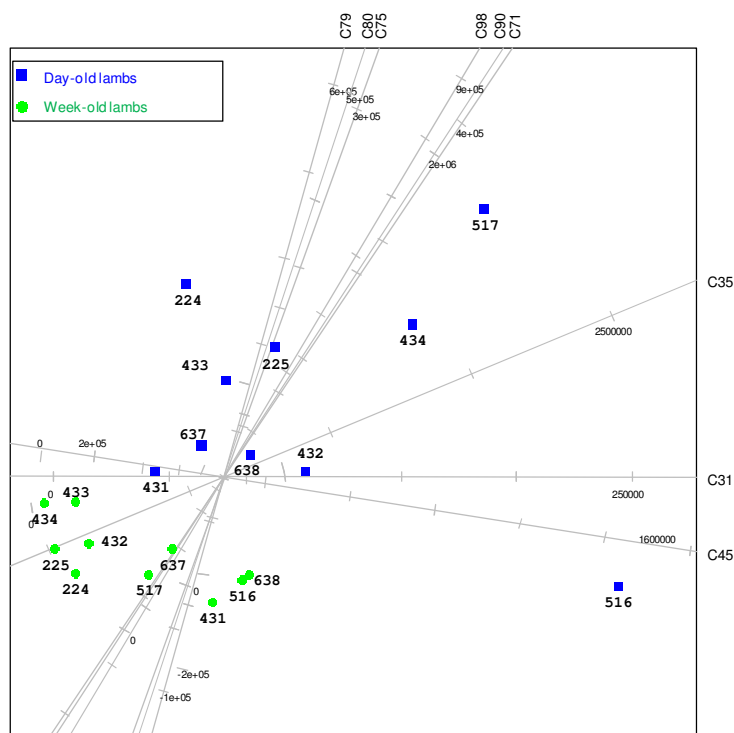


Fig. 4.29. PCA biplot for the wool volatiles collected from day-old lambs (blue) and from the same lambs a week after birth (green).

#### 4.1.4 Conclusions regarding the quantitative composition of the wool

A large number of volatile constituents belonging to a wide variety of compound classes were identified in the headspace gas of wool collected from 16 twins (32 lambs) in 2007, and three twins together with two single-born lambs in 2009. These volatiles were present in all the wool samples investigated, albeit in varying concentrations. Certain constituents were always present in high concentrations, and in total accounted for the larger part of the wool volatiles. The major constituents, with regard to percentage peak area, of the wool volatiles of day-old lambs collected in 2007 and 2009, in order of decreasing percentage peak areas, are ethyl tetradecanoate (**C88**), nonanal (**C27**), 6,10,14-trimethyl-2-pentadecanone (**C93**), tetradecanal (**C73**), pentadecanal (**C81**) and hexadecanoic acid (**C104**). The major constituents of the wool volatiles of week-old lambs correlate well with the constituents identified in the wool of day-old lambs, with only a few slight quantitative differences. These constituents are nonanal (**C27**), tetradecanal (**C73**), 6,10,14-trimethyl-2-

---

---

pentadecanone (C93), heptanal (C8), dodecanoic acid (C69), octanal (C19) and dodecanal (C57). Data with respect to the quantitative composition of the volatile organic material associated with wool samples collected during the lambing seasons of 2007 and 2009 are given in Table 4.3.

Three aspects of kin recognition in sheep were investigated. First, the genetic influence on the odour profiles of individual lambs was investigated by assessing the qualitative and quantitative similarity between the odour profiles of twins in comparison with those of randomly selected lambs by analysing the wool volatiles of the lambs. PCA biplot analyses were carried out and twins were grouped together according to the quantitative composition of the wool volatiles. Permutation tests showed that the pairing of lambs according to the composition of their wool is statistically significant, and that twins thus have odour profiles that are more similar than the odour profiles of other randomly selected pairs of non-twin lambs. It was shown that the lambs born of the same ewes in different years do not possess odour profiles that are more similar than the odour profiles of lambs born of different ewes.

Second, the change in the odour profiles of selected lambs was studied over a seven-day period. It was found that changes in the odour take place in a similar manner in all the lambs born during the lambing season of 2007. Thus, in general, compounds of lower mass, such as the group of aldehydes, for example, as well as the long-chain compounds, such as carboxylic acids and esters, became less prominent over the seven-day period. The calculated *P*-value of 0.0099 indicated that the pairing of twin lambs according to the quantitative composition of their wool volatiles is statistically significant and is not determined by chance. Interestingly, by making use of multivariate analyses analysis, it was found that not only did the odour profiles change, they also became increasingly similar over time from day 1 to day 7 after birth. For as long as a ewe makes use of olfactory recognition as her main form of identification of her lamb, her olfactory system has to continuously adapt to the change of the odour of the lamb and she has to continue learning the odour of her lamb.

Third, the possibility that maternal labels could be involved in the recognition of a lamb by its mother, in other words, the possibility that the lamb's odour is totally or partially determined by chemical compounds that are transferred in some way from the ewe to the lamb, was briefly investigated. The wool collected from the forehead and the abdomen of a ewe was compared with the wool collected from the forehead of her lamb and it was found that there was not any appreciable transfer of maternal labels from the ewe to the lamb.

## **4.2 Quantitative Analysis of Volatile Organic Fraction of the Amniotic Fluid**

### **4.2.1 Introduction**

During the sensitive period, lasting only a few hours after birth, the selective bond between ewe and lamb is formed, and it is during this time that the lamb's odour profile is embedded in the ewe's memory. The importance of amniotic fluid in maternal behaviour is well documented. In this part of the current study we set out to investigate the role that amniotic fluid could possibly play in the semiochemical communication between ewe and lamb.

### **4.2.2 Quantitative composition**

As mentioned in § 3.1.2.2 and § 3.1.3.2, the same sample group was used for the collection of wool and amniotic fluid samples in 2007. Amniotic fluid samples were collected from the same sample group of ewes that was used in the research on the role of the wool volatiles in kin recognition in sheep. Initially, methyl hexanoate, methyl undecanoate and methyl hexadecanoate, which are not present in the natural material, were added to the amniotic fluid as internal standards and the VOCs of the fluid were extracted once with a small volume of only 1 ml TBME. As in the case of the quantitative analyses of the VOCs in the wool samples (§ 4.1.2), better results were obtained by using synthetic analogues of the VOCs as external standards.

The high viscosity of the amniotic fluid precluded the quantitative extraction of the volatiles with such a small volume of solvent. However, faced with the choice between quantitative extraction and the risk of losing some volatile material during the evaporation of larger volumes of TBME, extraction with a small volume of solvent was preferred. This is because the accurate determination of the relative ratio in which the volatile compounds are present in the fluid was considered to be the main objective of this part of the research, and not the determination of the absolute quantities of the volatile constituents of the fluid. The average relative concentrations of the VOCs present in all of the 32 amniotic fluid extracts are given in Table 4.3. Relative percentage peak areas, derived from GC-MS results, are used in the graphical presentations and discussions below. Constituents present in concentrations lower than 0.001% of the total peak area were not considered in this investigation.

Approximately 78% of the total extracted organic compounds of the amniotic fluid of Döhne Merino ewes, taken as an average of the 32 samples analysed, consists of the six constituents: cholest-5-en-3 $\beta$ -ol (**C124**) (44%), hexadecanoic acid (**C104**) (12%), 9-octadecanoic acid (**C111**) (10%), two unidentified steroids (**C123**) (5%) and (**C121**) (4%), and octadecanoic acid (**C112**) (3%) (Fig. 4.30).

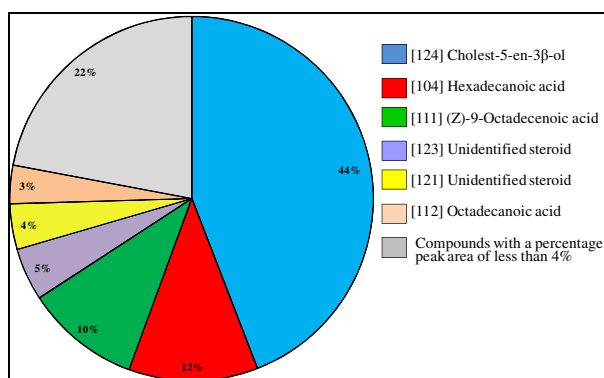


Fig. 4.30. Quantitative composition (average percentage peak areas) of the volatile organic fraction of the amniotic fluid collected from the bodies of Döhne Merino lambs during the lambing season of 2007.

---

---

Of these constituents only hexadecanoic acid (**C104**) was also identified as a major constituent of the wool volatiles collected from day-old lambs in 2007 (§ 4.1.2.1). Hexadecanoic acid and octadecanoic acid have previously been identified in lanolin. None of the major constituents present in the amniotic fluid were present in high percentages in the wool collected from week-old lambs. A more detailed analysis of the quantitative data is given in Figs. 4.31 and 4.32.

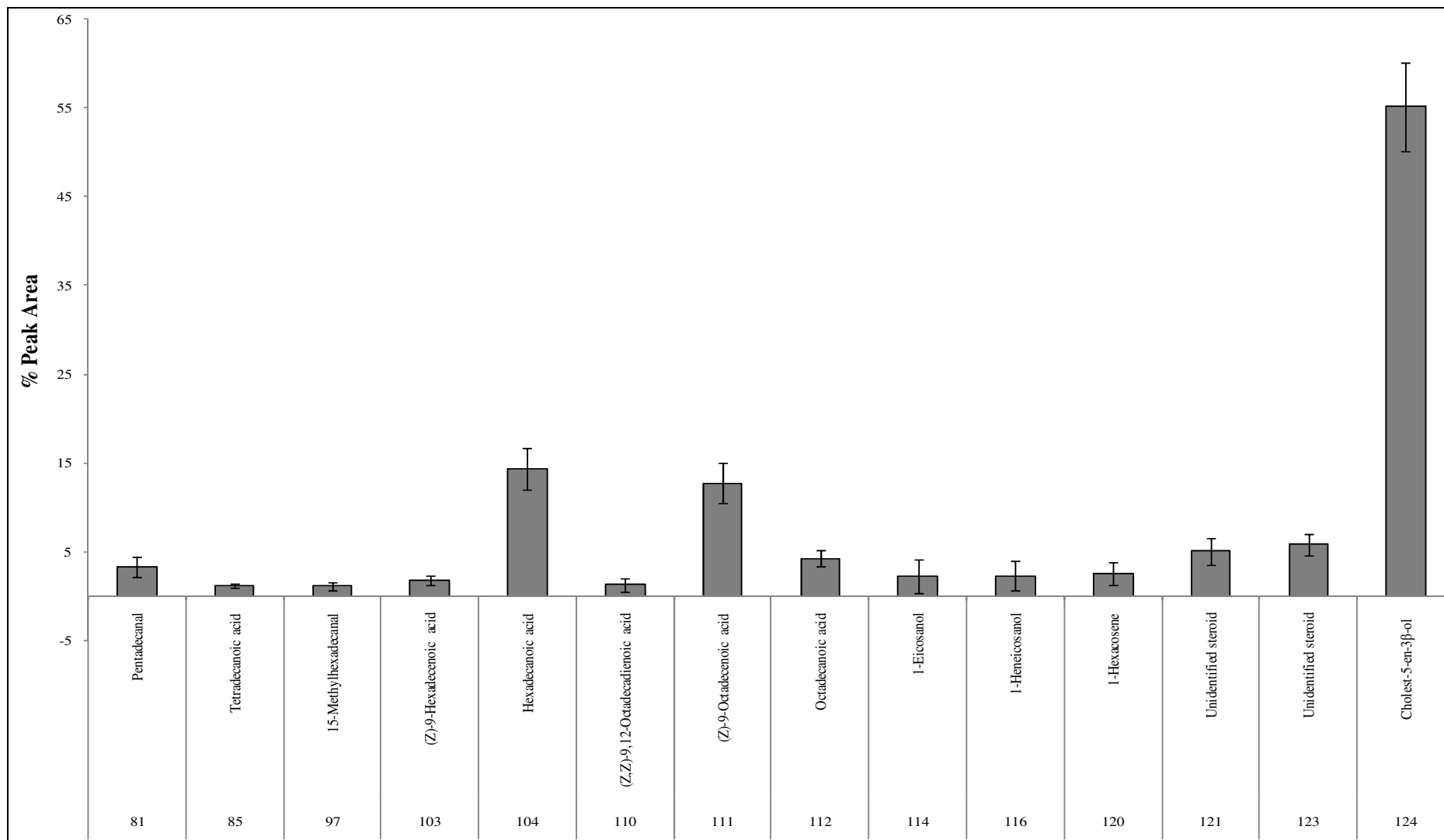


Fig. 4.31. Volatile constituents present in percentages larger than 1% of the volatile organic material extracted from the amniotic fluid that was collected from the bodies of newborn lambs during the lambing season of 2007. Constituents are shown in order of elution from Column A and constituent numbers are indicated on the x-axis.

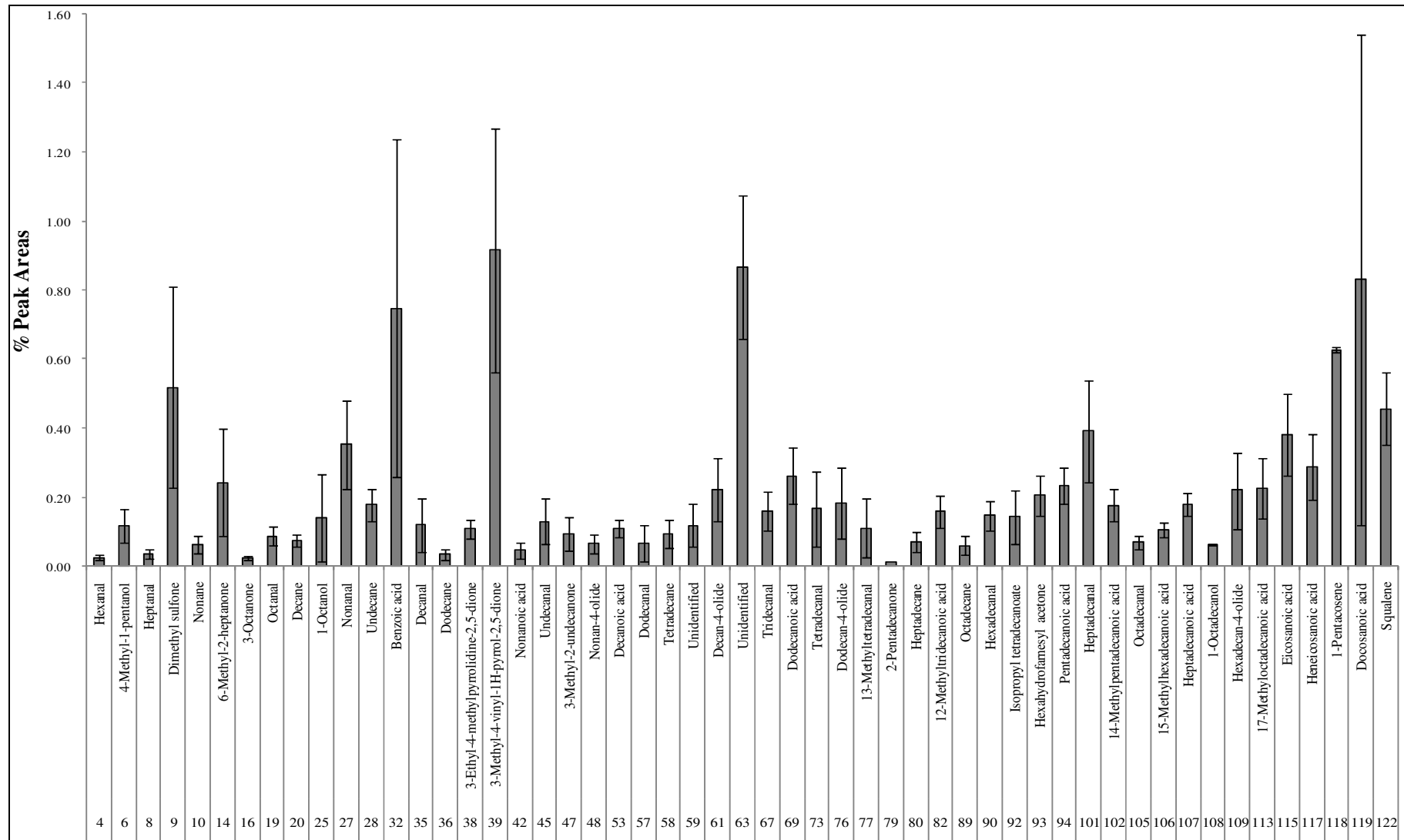


Fig. 4.32. Volatile constituents present in percentages smaller than 1% of the volatile organic material extracted from the amniotic fluid collected from the bodies of newborn lambs during the lambing season of 2007. Constituents are shown in order of elution from Column A and constituent numbers are indicated on the x-axis.



### 4.2.3 Statistical analysis

As previously mentioned in § 4.1.3, multivariate analysis methods were used to obtain meaningful interpretations of datasets that contain many variables and samples, such as the results of quantitative analyses of amniotic fluid samples.

#### 4.2.3.1 Comparison of the amniotic fluid composition collected from twin lambs

Data obtained in quantitative analyses of amniotic fluid samples collected during the lambing season of 2007 from 32 lambs (16 twins) born of 16 ewes, representing 9.69% of the ewe flock, were subjected to statistical analysis. Of the 70 identified constituents discussed in Chapter 3, only those present in 20% or more of the amniotic fluid samples were considered as variables for statistical analysis, which reduced the number of constituents to 48. The chromatographic data used for multivariate analyses were characterised by a large number of variables, in this case the peak areas of the identified constituents ( $n \leq 48$ ) compared to the number of sample units, and the number of amniotic fluid extracts ( $n = 32$ ). The peak areas were normalised across all samples to produce variables that could be compared with one another (Kowalski and Bender, 1972).

From an intra-twin comparison of the TICs of the extracts of the amniotic fluid samples collected from the twin lambs, US-2007-0049 and US-2007-0050 (Figs.4.33 and 4.34), it is clear that although they are not identical in all respects, there are many points of similarity between these two TICs. A similar relationship exists between the TICs, depicted in Figs.4.35 and 4.36, of the amniotic fluid volatiles collected from the twin lambs US-2007-0431 and US-2007-0432. An inter-twin comparison of, for example, the TICs depicted in Figs.4.33 and 4.35 reveals many points of difference between the amniotic fluid volatiles collected from the bodies of the respective lambs.

In Chapter 3 it was pointed out that many of the constituents identified in the amniotic fluid samples were present in less than 30% of the samples collected from the sample group of experimental animals. The constraints on the design of a semiochemical code for the type of kin recognition under discussion are such that a large number, and possibly also a wide variety of compound classes could be necessary to create a unique odour profile for each lamb (Alberts, 1992; Schaefer *et al.*, 2002; Singer *et al.*, 1997).

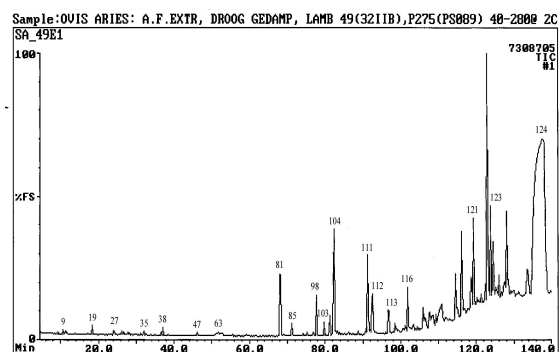


Fig. 4.33. TIC of the TBME extract of the amniotic fluid collected from the body of lamb US-2007-0049.

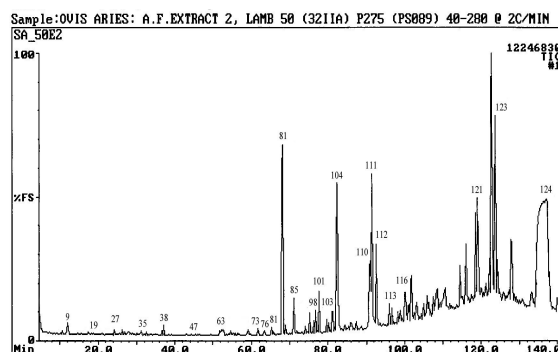


Fig. 4.34. TIC of the TBME extract of the amniotic fluid collected from the body of lamb US-2007-0050.

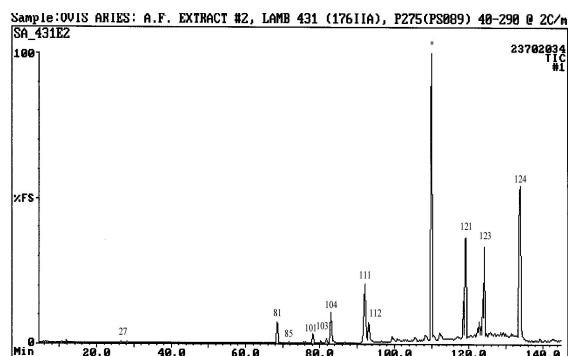


Fig. 4.35. TIC of the TBME extract of the amniotic fluid collected from the body of lamb US-2007-0431.

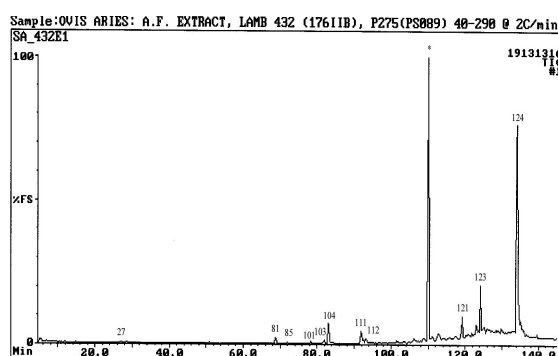


Fig. 4.36. TIC of the TBME extract of the amniotic fluid collected from the body of lamb US-2007-0432.

The number of compounds and compound classes that are used could largely depend on the tolerances at which the ewe's olfactory system operates. It was thought unlikely that compounds present in less than 30% of the collected samples could be a part of the flock of lambs' olfactory cue, and a decision was made to exclude all compounds present in less than 70% of the samples in the investigation. This considerably reduced the number of compounds that could be active components of the pheromone, and this in turn cast doubt on the amniotic fluid's role as a source of the unique olfactory cue. Additional compounds identified in subsequent analyses of the wool volatiles were added to the dataset, which accounts for the presence of some compounds, although they were only found in a few amniotic fluid samples. Multivariate analysis was used to assess whether the quantitative composition of the TBME extracts of the amniotic fluid of a pair of twins is more similar than the composition of the TBME extracts of two randomly selected non-twin lambs.

Permutation tests were carried out to determine whether the amniotic fluid extracts of the twin lambs can be grouped according to their quantitative composition. A  $P$ -value of 0.0268 was obtained for the permutation test, indicating that the pairing of the twin lambs according to the quantitative composition of their amniotic fluid is statistically significant. The permutation distribution in the form of a histogram is shown in Fig. 4.37 and the calculated  $P$ -value of 0.0268 is indicated by the red line. Although the  $P$ -value is of statistical significance, the distance of the red line from the centre of the histogram in Fig. 4.37 is not as far removed as it was in the case of the  $P$ -value calculated for the wool odour profiles of twin lambs (Fig. 4.21). This indicates that the amniotic fluid of twin lambs are not as similar in composition as the wool of twin lambs.

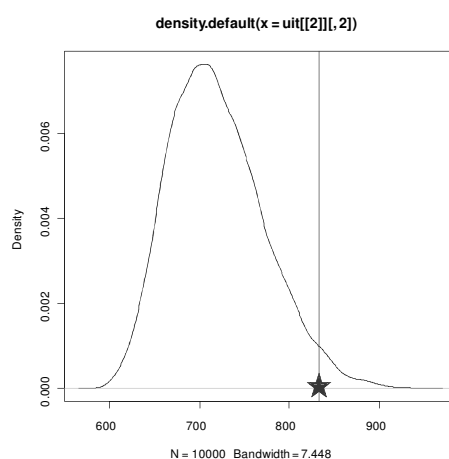


Fig 4.37. Permutation distribution showing the differences in the composition of the amniotic fluid of twin lambs compared to the rest of the group.

Given that 48 constituents were considered, the results were presented in the form of a biplot to provide insight into the multivariate character of the amniotic fluid composition. Only the 14 axes that best represent the predictivity in the amniotic fluid are displayed in the PCA biplot in Fig. 4.38. Each dot represents an amniotic fluid sample collected from the body of a newborn lamb and extracted with TBME. The last three digits of the lambs' numbers (as given in Table 4.1) are indicated on the biplot. The quality of display for the PCA biplot in Fig. 4.38 is 46%. The connecting black lines in the biplot in Figs. 4.38 and 4.39 indicate the positions of the 16 experimental twins. The 14 constituents that best represent the total variance in the amniotic fluid dataset with predictivities of higher than 0.600, in order of decreasing predictivity, are dodecane

(C36), heptadecane (C80), hexadecanal (C90), pentadecanal (C81), hexadecan-4-olide (C109), 12-methyltridecanoic acid (C82), decanal (C35), heptadecanal (C101), undecane (C28), tridecanal (C67), undecanal (C45), (*Z*)-9-octadecenoic acid (C111), nonane (C10) and tetradecanal (C73). Constituents C81, C82, C90 and C101 are a highly correlated group of variables in the biplot depicted in Figs.4.38 and 4.39. In these biplots the majority of the lambs are concentrated in the encircled area in the plot, indicating that all of these amniotic fluid samples have similar qualitative and quantitative compositions. Although some of the twin lambs are situated close to one another, large intra-twin distances separate the majority of the lambs. This suggests that the quantitative composition of the amniotic fluid of a pair of lambs is generally not more similar than the amniotic fluid of the rest of the group.

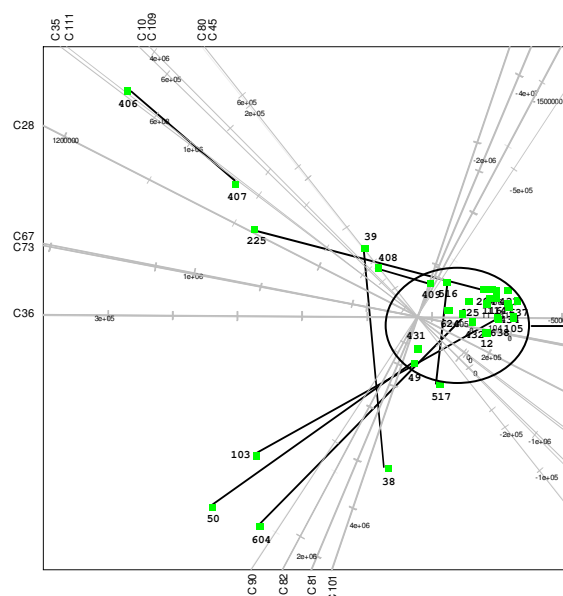


Fig. 4.38. PCA biplot of the amniotic fluid dataset displaying only the axes that best represent the variation in the amniotic fluid composition.

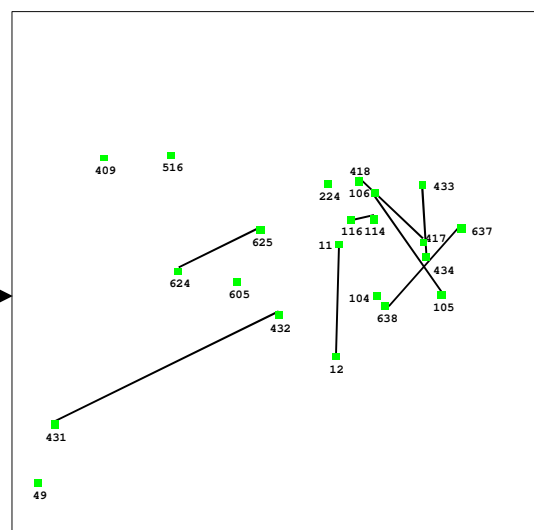


Fig. 4.39. Section of the PCA biplot depicted in Fig. 4.38 displaying the lambs in the concentrated area.

#### 4.2.3.2 Difference between the amniotic fluid compositions of first- and second-born lambs

During the collection of the amniotic fluid it was noticed that the colour of the amniotic fluid ranges from a colourless or whitish liquid, to progressively darker hues, ranging from shades of yellow to dark brown (Figs. 4.40 and 4.41). It was also noticed that the colour change in the amniotic fluid correlates with the duration of the lambing process. The longer a ewe takes to give birth to her lamb, the darker the colour of the

amniotic fluid becomes (John Morris, personal communication, 2009). This observation led to an investigation into the differences in the qualitative and the quantitative composition of the amniotic fluid collected from the first-born lamb compared to that of the second-born. The constituents present in 27 or more amniotic fluid samples (84%) were considered and the resulting  $\alpha$ -bagplot is shown in Fig. 4.42.



Fig. 4.40. Newborn lamb covered with mostly colourless amniotic fluid, with a slight discolouration at the lamb's hind quarters.



Fig. 4.41. Newborn lamb covered with yellow amniotic fluid.

An  $\alpha$ -bagplot can be defined as a contour enclosing the exact innermost  $\alpha\%$  of samples in a bivariate scatter plot, visualising the central tendency and correlation present in the data (Gardner, 2001). The open red square and the open green circle (in Figs.4.42 and 4.43) represent the mean of the peak areas of the VOCs of the amniotic fluid of the first- and second-born lambs, respectively. The area encircled by the red line indicates the position of the innermost 95% of the amniotic fluid samples of the first-born lambs and the area encircled by the green line the innermost 95% of the amniotic fluid samples of the second-born lambs. It can clearly be seen that the composition of the amniotic fluid of the first-born lambs differs substantially from that of the second-born lambs. In Fig. 4.43 connecting black lines indicate the position of three twins. In these three pairs of lambs, the total shift in the composition of the amniotic fluid from the first-born to second-born lamb is more accentuated.

The constituents that are present in 84% or more amniotic fluid samples in Fig. 4.42 are decane (C20), nonanal (C27), undecane (C28), unidentified constituent (C63), pentadecanal (C81), tetradecanoic acid (C85), 15-methylhexadecanal (C97), (Z)-9-

hexadecenoic acid (**C103**), hexadecanoic acid (**C104**), hexadecan-4-olide (**C109**), (*Z*)-9-octadecenoic acid (**C111**), octadecanoic acid (**C112**), unidentified steroid (**C121**), unidentified steroid (**C123**) and cholest-5-en-3 $\beta$ -ol (**C124**).

In Fig. 4.42 it can be seen that the amniotic fluid of the majority of the first-born lambs contains higher amounts of constituents **C20**, **C27**, **C28**, **C63**, **C109**, **C111**, **C112**, **C121** and **C123**, than the second-born lambs. The amniotic fluid of second-born lambs contains low amounts of the above-mentioned compounds and relatively high amounts of **C85**, **C97**, **C103** and **C104**. The amniotic fluid of first-born and second-born lambs contains relatively similar amounts of **C81** and **C124**.

Since a large number of aldehydes were identified in the amniotic fluid and in the headspace of the wool, the five additional aldehydes octanal (**C19**), decanal (**C35**), tridecanal (**C67**), hexadecanal (**C90**) and heptadecanal (**C101**), present in the amniotic fluid of 18 lambs or more (56%), were added to the dataset to produce the  $\alpha$ -bagplot shown in Fig. 4.43.

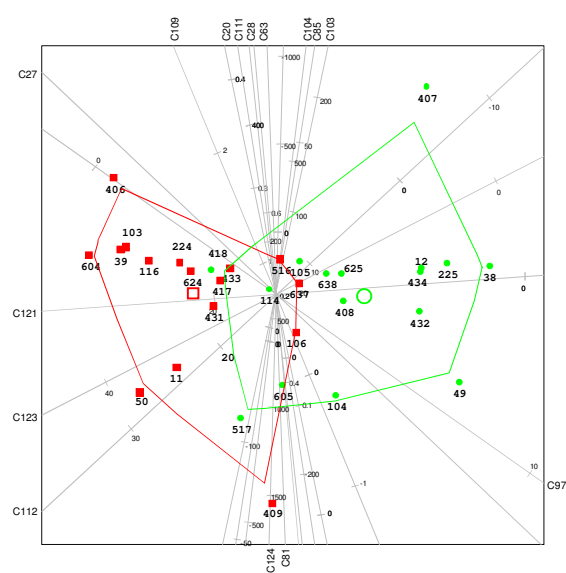


Fig. 4.42. 95%-Bagplot comparing the amniotic fluid of the first-born lambs (red) and second-born lambs (green). Squares and dots represent lambs and are indicated by the last three digits of the lambs' US numbers.

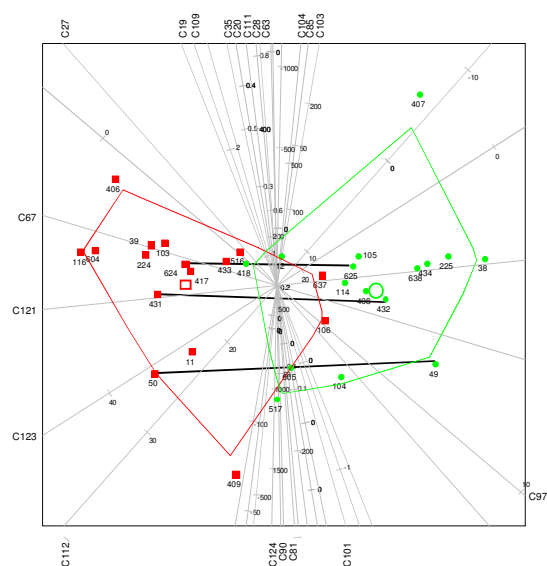


Fig. 4.43. 95%-Bagplot comparing the amniotic fluid of the first-born (red) and second-born (green). Five aldehydes have been added to the dataset depicted in Fig. 4.42. The positions of three twins are shown with black intra-twin connecting lines.

The basic profile of the bag plot was unchanged by the addition of additional aldehydes. If only the aldehydes are considered, it can be seen that the amniotic fluid of the first-born lambs contains higher amounts of the volatile aldehydes **C19**, **C27**, **C35** and **C67**. Second-born lambs contain low amounts of these compounds, and relatively higher amounts of the less volatile aldehydes **C81**, **C90**, **C97** and **C101**.

#### 4.2.4 Conclusions regarding the quantitative composition of the amniotic fluid

The TBME extracts of the collected amniotic fluid samples contain only relatively few volatile constituents present in low concentrations. A total of 76% of the extracted organic compounds consists of five constituents, of which cholest-5-en-3 $\beta$ -ol (**C124**) is present in the highest relative concentration.

In an investigation into the intra- and inter-twin similarities or differences in the qualitative and quantitative composition of the collected amniotic fluid samples, a *P*-value of 0.0268 indicated that the similarity of the composition of the extracts of a pair of twin lambs is of statistical significance. However, large intra-twin distances in Fig. 4.38 indicate that the amniotic fluid samples of twins are not particularly similar.

An investigation into the possibility that the amniotic fluid undergoes a process of change or degradation during the birth process revealed that the composition of the amniotic fluid continually changes in a similar fashion for all lambs, and that the volatile compositions of the amniotic fluids of first- and second-born twin lambs are quite different. This result put paid to the idea that the unique odour profile of the lambs could originate in the amniotic fluid, which at an earlier stage seemed to be a logical conclusion, based on the observation that all the constituents identified in the amniotic fluid were also present in the wool.

Given that the number of constituents identified in the amniotic fluid is much smaller than the number of constituents identified in the wool, and given that different analytical methods were used to analyse the volatiles in quite different matrices, an attempt was not made to carry out a rigorous multivariate comparison of the qualitative and quantitative compositions of the wool and amniotic fluid volatiles.

**Table 4.3: Average masses (ng) in which the VOCs are present in the amniotic fluid and the wool collected from Döhne Merino lambs during the lambing seasons of 2007 and 2009**

Comp. No.	Compound	Wool 2007	Wool 2009	Amniotic Fluid 2007
		Average mass (ng/70 mg wool; n = 32) <sup>a</sup>	Average mass (ng/70 mg wool; n = 8) <sup>a</sup>	Average mass (ng/1 g fluid; n = 32) <sup>b</sup>
1	3-Methylpentanal	8.65 (1.45) <sup>c</sup>	2.04 (0.32)	
2	1-Pentanol	4.52 (1.07)	3.09 (0.55)	
4	Hexanal	49.91 (5.68)	9.98 (1.94)	1.83 (0.32)
5	2-Methylpyrimidine	15.34 (5.06)	1.59 (0.39)	
6	4-Methyl-1-pentanol	17.03 (1.86)	8.15 (1.36)	12.57 (2.41)
8	Heptanal	66.36 (6.20)	16.27 (3.02)	1.40 (0.24)
9	Dimethyl sulphone		18.35 (5.46)	92.58 (18.91)
10	Nonane	2.16 (0.75)		2.40 (0.71)
11	2,5- Dimethylpyrimidine	40.41 (18.03)	4.09 (1.83)	
13	Benzaldehyde		4.62 (1.02)	
14	6-Methyl-2-heptanone	71.55 (7.74)	13.53 (2.54)	41.95 <sup>d</sup>
15	1-Heptanol	4.10 (0.61)	1.64 (0.34)	
16	3-Octanone	7.45 (0.75)	2.18 (0.50)	10.71 (4.84)
17	2-Octanone	8.44 (1.41)	5.84 (2.03)	
18	2-Pentylfuran	3.36 (0.32)	1.54 (0.16)	
19	Octanal	62.57 (6.82)	17.97 (3.34)	5.42 (1.49)
20	Decane	1.74 (0.40)		2.80 (0.46)
22	Phenylacetaldehyde		22.99 (2.50)	
23	( <i>E</i> )-2-Octenal	10.77 (1.40)	3.38 (0.76)	
24	7-Methyloctanal	5.60 (1.09)	8.38 (1.77)	
25	1-Octanol	17.26 (1.94)	1.83 (0.27)	
26	2-Nonanone	4.88 (0.92)	5.61 (0.71)	
27	Nonanal	303.09 (23.06)	62.88 (9.28)	17.66 (2.66)
28	Undecane	1.20 (0.17)	0.43 (0.09)	6.12 (0.78)
29	<i>N</i> -methyl-2-piperidinone	76.55 (23.69)	13.99 (3.00)	
30	( <i>E</i> )- 2-Nonenal	13.80 (1.66)	6.12 (0.88)	
31	8-Methylnonanal	6.20 (1.68)		
32	Benzoic acid			86.14 (30.49)
33	1-Nonanol	6.36 (1.15)	3.51 (0.44)	
34	2-Decanone	4.99 (0.85)	2.11 (0.49)	
35	Decanal	46.52 (5.29)	21.74 (2.85)	7.37 (2.39)
36	Dodecane	1.69 (0.21)	0.41 (0.09)	1.58 (0.36)
37	3-Ethyl-4-methyl-1H-pyrrole-2,5-dione		5.81 (2.11)	
38	3-Ethyl-4-methylpyrrolidine-2,5-dione		1.51 (0.40)	13.86 (6.00)
39	3-Methyl-4-vinyl-1H-pyrrole-2,5-dione		8.09 (1.69)	173.52 (80.89)
40	( <i>E</i> )-2-Decenal	13.94 (2.65)	6.81 (2.05)	
41	9-Methyldecanal	4.54 (1.06)	0.76 (0.16)	



Table 4.3 (continued)

Comp. No.	Compound	Wool 2007	Wool 2009	Amniotic Fluid 2007
		Average mass (ng/70 mg wool; n = 32) <sup>a</sup>	Average mass (ng/70 mg wool; n = 8) <sup>a</sup>	Average mass (ng/1 g fluid; n = 32) <sup>b</sup>
42	Nonanoic acid	14.57 (10.02)	2.30 (0.59)	8.79 (3.43)
43	2-Undecanone	3.76 (1.32)	0.95 (0.18)	
44	1-Tridecene	0.68 (0.13)	0.39 (0.09)	
45	Undecanal	28.50 (5.58)	8.61 (0.89)	8.99 (5.62)
46	Tridecane	2.81 (0.31)	0.70 (0.06)	
47	3-Methyl-2-undecanone	1.71 (0.32)	0.75 (0.26)	3.73 (0.90)
48	Nonan-4-olide	3.39 (0.37)	1.40 (0.25)	1.47 (0.40)
50	( <i>E</i> )-2-Undecenal	7.95 (1.39)	2.64 (0.59)	
51	10-Methylundecanal	17.24 (3.75)	12.28 (2.67)	
53	Decanoic acid	14.53 (4.69)	3.38 (1.09)	31.03 (9.49)
54	2-Dodecanone	1.73 (0.22)	1.45 (0.36)	
55	1-Tetradecene	0.59 (0.10)		
56	6,10-Dimethyl-2-undecanone	1.49 (0.35)	1.54 (0.67)	
57	Dodecanal	86.84 (10.57)	27.70 (3.60)	4.85 (3.60)
58	Tetradecane	17.12 (13.07)	2.35 (0.27)	8.35 (3.92)
60	( <i>5E</i> )-6,10-Dimethyl-5,9-undecadien-2-one	6.48 (2.00)	2.23 (0.19)	
61	Decan-4-olide			7.78 (2.33)
62	( <i>E</i> )-2-Dodecenal	1.46 (0.51)		
65	2-Tridecanone	4.07 (2.10)	0.59 (0.13)	
66	1-Pentadecene	0.89 (0.14)	1.72 (1.01)	
67	Tridecanal	74.27 (13.14)	17.17 (2.42)	17.58 (3.57)
68	12-Methyltridecanal	8.20 (1.74)	3.84 (0.77)	
69	Dodecanoic acid	163.07 (59.30)		46.06 (12.60)
71	2-Tetradecanone	3.29 (0.51)	2.13 (0.38)	
72	Hexadecane	22.68 (14.61)	1.97 (0.16)	
73	Tetradecanal	150.61 (18.03)	16.94 (2.55)	13.73 (6.33)
75	1-Tetradecanol	3.67 (1.01)	1.10 (0.21)	
76	Dodecan-4-olide			4.54 (1.36)
77	13-Methyltetradecanal	12.26 (2.22)	1.44 (0.44)	9.98 (4.08)
78	( <i>E</i> )-2-Tetradecenal	11.98 (2.36)	0.93 (0.19)	
79	2-Pentadecanone	5.91 (1.18)	1.38 (0.32)	
80	Heptadecane	14.99 (8.99)	1.24 (0.16)	2.07 (0.70)
81	Pentadecanal	128.77 (15.75)	7.52 (1.58)	316.84 (104.96)
82	12-Methyltridecanoic acid			53.70 (15.63)
85	Tetradecanoic acid	135.85 (49.33)	10.71 (2.62)	433.81 (211.71)
86	14-Methylpentadecanal	14.87 (3.30)		
87	1-Pentadecanol	26.00 (12.44)	1.66 (0.28)	
88	Ethyl tetradecanoate	313.51 (96.70)	50.83 (16.10)	
89	Octadecane	10.80 (5.16)	1.16 (0.26)	2.89 (1.02)
90	Hexadecanal	28.32 (4.20)	2.32 (0.55)	12.25 (3.05)

Table 4.3 (continued)

Comp. No.	Compound	Wool 2007	Wool 2009	Amniotic Fluid 2007
		Average mass (ng/70 mg wool; n = 32) <sup>a</sup>	Average mass (ng/70 mg wool; n = 8) <sup>a</sup>	Average mass (ng/1 g fluid; n = 32) <sup>b</sup>
91	2,6,10,14-Tetramethyl-hexadecane	7.47 (2.37)	0.91 (0.24)	
92	Isopropyl tetradecanoate	78.66 (27.35)	1.51 (0.38)	6.31 (2.35)
93	6,10,14-Trimethyl-2-pentadecanone	276.33 (42.79)	30.92 (7.90)	19.22 (7.71)
94	Pentadecanoic acid		3.54 (1.13)	83.05 (29.00)
95	6,10,14-Trimethyl-2-pentadecanol	17.20 (4.23)	1.90 (0.66)	
97	15-Methylhexadecanal	20.63 (5.73)		84.00 (26.49)
98	1-Hexadecanol	9.12 (2.89)		
99	2-Heptadecanone	30.66 (11.52)	2.15 (0.42)	
100	Nonadecane	11.93 (6.17)	0.52 (0.24)	
101	Heptadecanal	13.85 (5.72)	1.79	31.93 (9.86)
102	14-Methylpentadecanoic acid			78.30 (33.21)
103	(Z)-9-Hexadecenoic acid			1080.43 (739.08)
104	Hexadecanoic acid	140.65 (41.28)	54.07 (12.25)	5080.10 (2283.69)
105	Octadecanal			10.40 (4.05)
106	15-Methylhexadecanoic acid			61.03 (22.52)
107	Heptadecanoic acid			94.92 (36.27)
108	1-Octadecanol			7.68 (3.07)
109	Hexadecan-4-olide			10.44 (3.46)
110	(Z,Z)-9,12-Octadecadienoic acid			240.34 (171.82)
111	(Z)-9-Octadecenoic acid			4843.91 (1731.50)
112	Octadecanoic acid			836.90 (289.79)
113	17-Methyloctadecanoic acid			121.13 (39.95)
114	1-Eicosanol			52.95 (11.93)
115	Eicosanoic acid			165.66 (79.53)
116	1-Heneicosanol			85.74 (13.56)
117	Heneicosanoic acid			156.24 (112.03)
118	1-Pentacosene			200.00 (32.68)
119	Docosanoic acid			197.02 (112.46)
120	1-Hexacosene			141.92 (62.86)
122	Squalene			91.92 (38.60)
124	Cholest-5-en-3 $\beta$ -ol			17890.08 (3830.90)

<sup>a</sup> Quantities present in the headspace of the 70 mg wool sampled at 70 °C

<sup>b</sup> Quantities present in the TBME extract of 1 g of amniotic fluid

<sup>c</sup> SEM indicated in brackets

<sup>d</sup> SEM can only be calculated for compounds present in more than one sample

---

---

## References

Alberts, A. C., “Constraints on the design of chemical communication systems in terrestrial vertebrates”, *Am. Nat.* **139**: S62–S89 (1992).

Dawson-Saunders, B. and Trapp, R. G., “Basic & Clinical Biostatistics”, Second Edition, Appleton & Lange, Connecticut (1994).

Gardner, S., “Extensiond of Biplot Methodology to Discriminant Analysis with Applications of Non-Parametric Principal Components”, Ph.D Thesis, University of Stellenbosch (2001).

Gardner-Lubbe, S., Le Roux, N. J. and Gower, J. C., “Measures of fit in principal component and canonical variate analyses”, *J. Appl. Statist.* **35**: 947–965 (2008).

Gower, J. C., “Analysis of distance for structured multivariate data and extensions to multivariate analysis of variance”, *Appl. Statist.* **48**: 505–519 (1999).

Gower, J. C. and Hand, D. J., “Biplots”, Chapman & Hall, London (1996).

Kowalski, B. R. and Bender, C. F., “Pattern recognition. A powerful approach to interpreting chemical data”, *J. Am. Chem. Soc.* **94**: 5632–5639 (1972).

Le Roux, N. J. and Lubbe, S., “Biplots and clustering for high dimensional paired data”, 11<sup>th</sup> Conference of the International Federation of Classification Societies (IFCS), University of Technology, Dresden, Germany, March 2009.

Lisovac, A. M. and Shooter, D., “Volatiles from sheep wool and the modification of wool odours”, *Small Ruminant Res.* **49**: 115–124 (2003).

Motiuk, K., “Wool wax acids: a review”, *J. Am. Oil Chem. Soc.* **56**: 91–97 (1979a).

---

---

Motiuk, K., “Wool wax alcohols: a review”, *J. Am. Oil Chem. Soc.* **56**: 651–658 (1979b).

Motiuk, K., “Wool wax hydrocarbons: a review”, *J. Am. Oil Chem. Soc.* **57**: 145–146 (1980).

Olivier, J. J., Shepherd Computerised flock-recording program, ARC Animal Improvement Institute, RSA (2003).

Romeyer, A., Porter, R. H., Poindron, P., Orgeur, P., Chesné, P. and Poulin, N., “Recognition of dizygotic and monozygotic twin lambs by ewes”, *Behaviour* **127**: 119–139 (1993).

Schaefer, M. L., Yamazaki, K., Osada, K., Restrepo, D., and Beauchamp, G. K., “Olfactory fingerprints for major histocompatibility complex-determined body odors II: relationship among odor maps, genetics, odor composition, and behaviour”, *J. Neurosci.* **22**: 9513–9521 (2002).

Schlossman, M. L. and McCarthy, J. P., “Lanolin and derivatives chemistry: relationship to allergic contact dermatitis”, *Contact Dermatitis* **5**: 65–72 (1979).

Singer, A. G., Beauchamp, G. K., and Yamazaki, K., “Volatile signals of the major histocompatibility complex in male mouse urine”, *Proc. Natl. Acad. Sci. USA* **94**: 2210–2214 (1997).

Wyatt, T. D., “Pheromones and Animal Behaviour. Communication by Smell and Taste”, Cambridge University Press, Cambridge (2003).

## CHAPTER 5

### BIOASSAYS

#### 5.1 Introduction

Bioassays were carried out during the final stage of this study to determine whether the adoption of alien lambs could be facilitated by making use of synthetic mixtures containing the VOCs identified in the cranial wool of lambs in the same qualitative and quantitative composition as in the wool of the experimental lamb used in a particular test. This is the most crucial part of an investigation into the phenomenon of kin recognition. As previously mentioned (§ 4.1.3), constituents singled out by multivariate analyses techniques as principal components are possibly, but not necessarily, the constituents regarded by a particular animal as important key role players in kin recognition. In the current literature, claims are made with regard to the possible role that constituents could play in kin recognition without providing substantiating evidence obtained from bioassays.

In the present study, bioassays were carried out during the lambing season of 2009, which, in the case of the Mariendahl flock, lasted for three weeks during the months of March and April. Twin-bearing ewes were used in most of the experiments and only one of the lambs of a twin was exchanged with an alien lamb. All bioassays were conducted in lambing pens or outside in an enclosed concrete-surfaced arena. Bioassays were carried out 24 h to 72 h after parturition. Alien lambs used in these experiments were matched in size as closely as possible to the ewes' own lambs and the experimental ewes' own lambs were taken well outside hearing range during experiments. Drastic measures have been used in several other studies to force ewes to accept alien lambs treated with various odours or synthetic materials. For example, ewes were tethered to prevent them from displaying their typical udder denial, which involved rapidly and constantly turning around in circles. In these studies persistent attempts at fostering alien lambs were continued over periods of up to 72 h (Alexander *et al.*, 1987, 1989). In the present study such drastic methods were not applied. An alien lamb was considered accepted if the lamb was accepted at the udder and

---

---

allowed to suckle and if the ewe directed no butts or butt attempts at the alien lamb for a period of five minutes after their first contact. If alien lambs were rejected by the ewe and the ewe had displayed aggressive behaviour towards the alien lamb, the experiment was immediately terminated and the alien lamb permanently removed from the experimental arena.

To prevent odour transfer from the alien lamb to the jackets or diffusion through the jackets during the five minutes that the bioassays lasted, alien lambs were fitted with disposable diapers and, in addition, the lambs' bodies were covered from their hind quarters to their shoulder blades with several layers of thin, low density polyethylene based plastic, and dressed in jackets of the various designs described § 2.8, and shown on Plates 5.1a and 5.1b. Hooded or hoodless jackets were made from white or grey cotton fleece cloth. The hoodless jackets covered the lambs' bodies from the tail to the back of the neck (Plate 5.1b), with openings for the legs. Hooded jackets covered the lambs' bodies as well as the neck and face, with openings for the ears, eyes, mouth and legs (Plate 5.1c). The jackets were fastened ventrally using safety pins.

## 5.2 Bioassays using Synthetic Mixtures

Having found that lambs, whether twins or single-born lambs, have unique odour profiles that differ from those of the lambs born to other ewes, and that twins have odour profiles that are almost identical (§ 4.1.1), the next step was to conduct bioassays in order to determine whether it would be possible to induce a ewe to accept an alien lamb treated with a synthetic version of her own lamb's odour.

Synthetic mixtures<sup>8</sup> consisting of the compounds that had been identified as wool volatiles were made up according to qualitative information obtained from analyses that had been done on wool samples collected in 2007. However, the final formulation of mixtures of synthetic compounds for behavioural bioassays in 2009 was determined in each case by the

---

<sup>8</sup> Some of the wool volatiles in Table 3.1 were not included in test mixtures because they were either unidentified or had not yet been synthesised when these bioassays were carried out.

---

---

results of a GC-MS analysis of the cranial wool of the lamb of the ewe involved in the bioassay, which was carried out within 24 h after the wool had been harvested.

For exploratory bioassays solutions containing compounds belonging to the same compound class were made up. The solution of the aldehydes was first sprayed onto a jacket (Plate 5.1d), the pentane was allowed to evaporate and the alien lamb was presented to a ewe. Using the same jacket, this procedure was repeated with solutions of the acids, esters and ketones and, finally, a solution of the alcohols and nitrogen-containing compounds. The ewe was most disturbed by the aldehyde mixture, and displayed the most interest in the mixture containing the alcohols and the nitrogen-containing compounds. The interest shown by the ewe when presented with the latter mixture could perhaps be attributed to the combined effect of all the identified wool volatiles. The ewe repeatedly sniffed at the lamb, while calling for her own lamb, turning around in circles and not allowing the alien lamb at the udder. The alien lamb was eventually rejected. A similar experiment, carried out with appropriately formulated mixtures (*vide supra*), also produced negative results.

It was noted that the jacket sprayed with synthetic analogues of all the wool volatiles did not smell like a lamb and it was thought that lanolin could have a certain modulating effect on the dissemination of compounds with different boiling points or belonging to different compound classes, and that this could be the reason for the atypical smell of the treated jacket. A commercial sample of lanolin was then purified by stripping it as far as possible of solvent residues, impurities and the volatile constituents of the lanolin. The purified lanolin was dissolved in pentane and sprayed on a jacket. Ewes showed interest in alien lambs dressed in this jacket by sniffing at them, but still did not accept the alien lambs ( $n = 3$ ). After establishing that lanolin alone was not effective in fostering alien lambs, lanolin was added to a jacket that had been treated with the synthetic mixtures. This experiment was repeated with a paper jacket treated in a similar fashion. The alien lambs were rejected in both experiments.

An important question that had to be answered was whether at 70 °C volatiles are released from the wool in the same quantitative ratio in which they evaporate from a lamb's

body under normal conditions. It was however thought that this is possibly not exceedingly critical. Although the lamb's body temperature remains constant under natural conditions, the animal's wool could be at a temperature considerably higher or lower than its body temperature. Nevertheless, 70 °C is much higher than the temperatures at which volatiles are released from the wool of a lamb living under normal climatic conditions. Thus, although sample enrichment at 40 °C during the development of analytical techniques for the present study (§ 3.1.3.3.1, Figs. 3.5 and 3.9) did not produce very useful results, SEP enrichment at 40 °C and GC-MS analysis were nevertheless carried out on a sample of a lamb's cranial wool to obtain qualitative and quantitative information that could be used to compose an appropriately formulated synthetic test mixture for bioassays. In the first bioassay with this mixture the alien lamb was not accepted. The lamb was removed from the ewe, lanolin was applied to the jacket that had been used, and the alien lamb was again presented to the ewe. She again responded negatively. The alien lamb was removed from the ewe and another alien lamb was fitted with a new jacket. In this case the synthetic mixture was applied to the jacket at twice the concentration at which the constituents were present in the analysed wool. Once again the ewe rejected the alien lamb.

### **5.3 Bioassays using Different Odour-Transfer Techniques**

The unsuccessful attempts at fostering lambs using synthetic mixtures raised questions about the physical properties of the semiochemical message between lamb and ewe, for example, about the volatility of the signal and its longevity (persistence) under normal conditions. It was decided that more background information could perhaps be obtained by conducting experiments on the transfer of the odour from a lamb to, for example, a fleece jacket.

#### **5.3.1 Odour-impregnated jackets**

Price *et al.* (1984) showed that lambs can be fostered by means of odour transfer using nylon stockinettes. Lambs were fitted with stockinettes for 72 h, removed from the ewe's own lamb, and placed turned inside-out on an alien lamb, and then presented to the



ewe. A total of 38% of the alien lambs were immediately accepted and another 50% percent were accepted over a further period of 36 h. These results were confirmed in an exploratory experiment carried out during the 2009 lambing season by leaving jackets on lambs ( $n = 7$ ) for 72 h and dressing alien lambs with the jackets turned inside-out. All ewes accepted the alien lambs at the udder. The acceptance of alien lambs confirmed that this method of odour transfer from an own lamb to an alien lamb is possible, as reported in the literature (Price *et al.*, 1984).

The volatile constituents present in one of these jackets used in the successful fostering experiments were analysed using a SEP50 at 40 °C (Fig. 5.1). The results are given in Table 3.1. No new constituents were identified in the headspace of the jacket. All the constituents present in the jacket used in the positive bioassay test had thus already been included in the synthetic mixtures discussed in § 5.2.

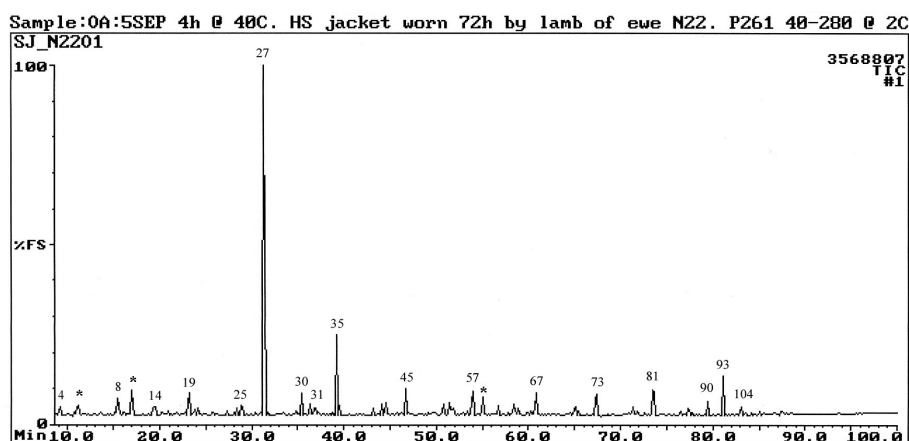


Fig. 5.1. TIC obtained by GC-MS analysis of the headspace volatiles SEP-enriched at 40 °C from a jacket worn by a Döhne Merino lamb and used in a successful fostering experiment.<sup>9</sup>

\* Siloxanes

The change in the odour profile of a lamb during the course of one week is discussed in § 4.1.2.2 and § 4.1.3.2, where it has been pointed out that although the odour profile of each lamb is constantly changing, its olfactory signature remains unique. For as long as a ewe makes use of olfactory recognition as the main form of identification, the ewe apparently

<sup>9</sup> All the TICs depicted in this chapter were obtained in GC-EIMS analyses using Column A and the analytical conditions given in Chapter 2.

---

---

has to continuously monitor and keep up with the changing odour profile of her lamb or lambs. To determine how long a ewe can be separated from her lamb before the difference between the odour profile embedded in her memory and the actual odour profile of her lamb at a later stage has increased to such an extent that she will reject the lamb, the following experiment was carried out.

Jackets were left on the lambs of five ewes for 72 h, after which five alien lambs dressed in the jackets were presented to these ewes. The alien lambs were all accepted and the jackets were immediately removed and stored at  $-18\text{ }^{\circ}\text{C}$  on day 1. Alien lambs were fitted with two of these jackets in similar bioassays on day 4 (three days later). The alien lambs were accepted at the udder. Two further jackets were used in a similar fashion on day 7. The alien lambs were rejected. The alien lamb on which the fifth jacket was used was also rejected on day 10. This indicated that a ewe can still recognise the odour profile imprinted on the jacket as that of her own lamb on day 4. However, after six days the difference between the odour of her lamb that she had been continually monitoring during the six days had changed so much that she no longer recognised the odour profile on the jacket as that of her own lamb. At this point it was argued that the ewe would probably accept the alien lamb dressed in the jacket after a longer period if the ewe could not monitor the changing odour of her own lamb. It was decided not to further interfere to this extent with the breeding programme at Mariendahl.

### 5.3.2 Wool of a lambs

In a single experiment, the largest possible quantity of wool (*ca.* 8 g) was sheared from the head and body of a day-old lamb and stitched to a jacket using a layer of cotton gauze to keep the wool in place. An alien lamb was fitted with the jacket and presented to the ewe. The ewe rejected the lamb.

### 5.3.3 Extracts of the wool of lambs

The quantitative differences between the organic volatiles extracted with TBME from

---

---

lambs' wool and those SEP-extracted from the headspace of the wool have been described in § 3.1.3.3.3. The possibility of using extracts of the wool volatiles in suitable solvents in bioassays was therefore considered. Exploratory experiments were carried out in which *ca.* 2 g wool was collected from the foreheads of three day-old lambs and the three crops of wool individually Soxhlet-extracted with DCM, TBME and pentane, respectively, for 1.5 h. The extracts were evenly applied to three jackets. The solvent was allowed to evaporate before bioassays were carried out as usual using three alien lambs. The lambs were all rejected.

The ability of a cloth jacket to release the individual extracted constituents in the same ratio as wool was considered. The possibility that a jacket made from brown paper could yield better results was therefore investigated. Wool (2 g) was collected from the forehead of a day-old lamb, Soxhlet-extracted with DCM and evenly applied to a brown paper jacket, which was then fitted to an alien lamb. Plate 5.1e shows the alien lamb fitted with the brown paper jacket as it was presented to a ewe. Although the ewe displayed initial interest in the lamb, she did not allow the lamb to suckle. Purified lanolin was applied to the brown paper jacket that had been treated with the DCM extract and the fostering test was repeated with the same ewe. The alien lamb was rejected.

To increase the concentration of the wool extracts, *ca.* 8 g wool was sheared from the bodies of day-old lambs and Soxhlet-extracted with either pentane ( $n = 6$ ) or ethyl acetate ( $n = 1$ ) for 1.5 h. These extracts were evenly applied to jackets and the solvent allowed to evaporate before bioassays were carried out. In two instances, the wool sheared from the bodies of twin lambs were separately extracted with pentane and the extracts combined and evenly applied to a single jacket. In bioassays using the pentane ( $n = 5$ ) and ethyl acetate ( $n = 1$ ) extracts, the alien lambs were all rejected. In one special case, an alien lamb fitted with a jacket that was treated with a pentane extract was accepted by the ewe and allowed to suckle without any hesitation (Plate 5.1f). The lambs of this ewe had been wearing jackets for 72 h as part of another experiment. This probably conditioned her to the presence of lambs wearing jackets and this might have facilitated the acceptance of the alien lamb.

The role that proteins could possibly play in facilitating kin recognition was investigated. In one experiment the protein remaining on the wool after Soxhlet-extraction with pentane was extracted with water containing a detergent. A jacket that had been used in one of the above-mentioned unsuccessful fostering experiments (pentane extract) was coated with the protein extract, and bioassayed. The alien lamb was rejected. In another experiment, a jacket was coated with the pentane and protein extracts in the reverse order. The alien lamb wearing this jacket was also rejected by the ewe.

Small volumes of the extracts were concentrated as described in § 2.3.3.1 for subsequent GC-MS analysis. A TIC of one of these pentane extracts is shown in Fig. 5.2. On comparison of this pentane extract with a TBME extract (Fig. 5.3), the constituents previously identified in TBME extracts of wool samples were also identified in the pentane extracts, although, for some inexplicable reason, more impurities (*i.e.* siloxanes) were present in the pentane extract. As previously mentioned (§ 3.1.4.3), TBME extracted more material from the wool, however, pentane was preferred for the extraction of material that was used in bioassays because the careful evaporation of the TBME was an extremely slow process and, furthermore, TBME has such an unpleasant smell to the human nose that it was feared that it could interfere with the planned bioassays.

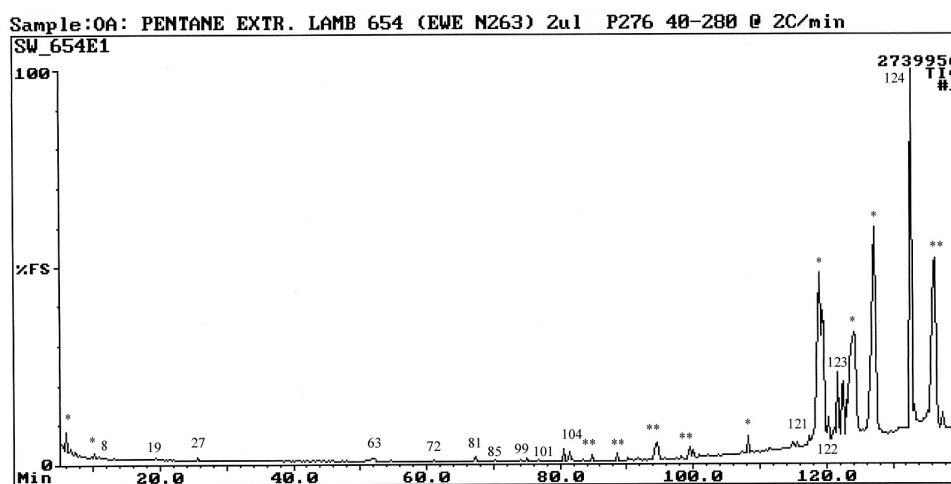


Fig. 5.2. TIC of a typical pentane extract of the wool of lamb US-2009-0654.

\* Siloxanes

\*\* Unidentified constituents commonly associated with lanonin

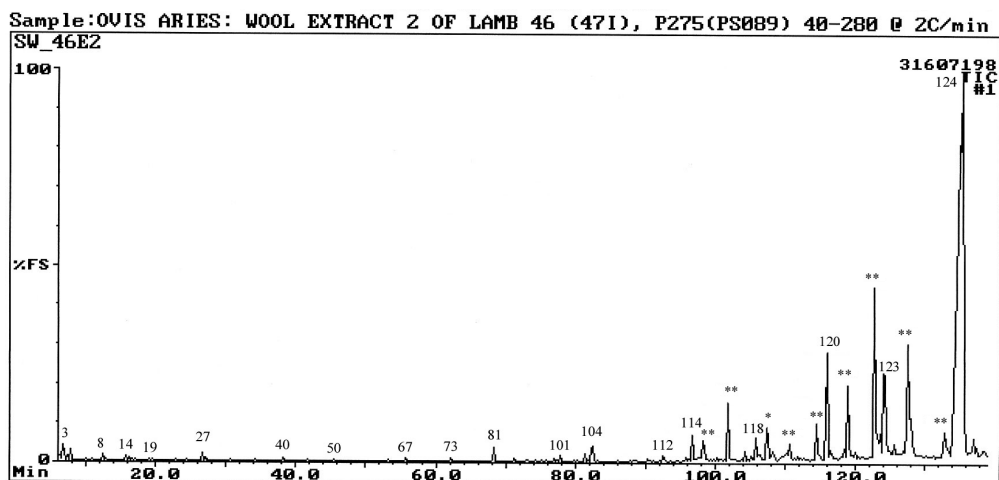


Fig. 5.3. TIC of a typical TBME extract of the wool of lamb US-2007-0046.

\* Siloxanes

\*\* Unidentified constituents commonly associated with lanonin

## 5.4 Conclusions

Bioassays were carried out to determine whether the adoption of alien lambs could be facilitated by making use of synthetic mixtures containing the VOCs in the same qualitative and quantitative composition as the wool of the experimental ewe's own lamb in a particular experiment. It became clear that it would not be a simple matter to facilitate add-on fostering in this manner because the composition of the VOCs released into the atmosphere from a lamb's body constantly changes under normal circumstances. This requires constant adjustment of the ewe's evaluation of the olfactory information, which in turn is the reason for the constant and sometimes frantic monitoring of a lamb's odour by its mother. On one hand, the changes in the composition over a period of six days discussed in §4.1.2.2 are surprisingly large, which could be used as an argument in favour of not attempting to formulate test mixtures reflecting the natural odour of the experimental lambs in the finest detail. On the other hand, the quantitative data that were used in the formulation of test mixtures might not have been accurate enough. Instead of GC-FID analyses, which give a more accurate picture of the quantitative composition of an analytical sample, GC-MS analyses had to be used in the limited time available to avoid the misidentification of the volatile compounds in the wool.

---

---

It was possible to achieve add-on fostering by dressing alien lambs in jackets that had been worn by ewes' own lambs for three days. One of these jackets was subjected to a headspace analysis using SEP50 sampling. All the VOCs identified in wool samples were found in the headspace gas of the jacket and had been appropriately included in the synthetic test mixtures.

By making use of jackets that had been worn by ewes' own lambs for 72 h and were subsequently stored at  $-18\text{ }^{\circ}\text{C}$  for ten days, it was found that three days after removal of a jacket from the lamb a ewe could still recognise the odour of her lamb on the jacket that her lamb has worn. However, after six days alien lambs fitted with the jackets were rejected. These results are in agreement with the results discussed in § 4.1.3.2 where it was shown that the odour of the lamb undergoes a significant change over a period of seven days and the results of this bioassay confirm that a ewe is continually learning the odour of her lamb during the period she uses olfactory stimuli as the main form of lamb recognition.

Many bioassays were carried with jackets treated with wool extracts in attempts at facilitating add-on fostering of alien lambs. In only one of these experiments did a ewe accept an alien lamb at the udder. A jacket treated with a pentane extract of the wool was used in this experiment. A possible explanation for the positive outcome of this experiment is discussed in Chapter 6.

Alien lambs dressed in jackets treated with lanolin, or with a combination of lanolin, synthetic VOCs or wool extracts were all rejected by ewes in bioassays carried out to establish whether the presence of lanolin could have a positive effect on the outcome of bioassays with synthetic or natural VOCs. At this exploratory stage it is still too early to reach any conclusions regarding lanolin's possible role in the phenomenon under investigation.

The role that proteins could possibly play in facilitating the fostering of alien lambs was investigated by either applying proteins extracted from wool samples to a jacket previously treated with a wool extract, or by first applying the extracted proteins to a jacket

---

---

and then the extract. As was the case in the experiments with lanolin, no effect could be observed.

The breeding program followed at Mariendahl precluded using the rams that fathered the 2007 lambs again in the 2009 lambing season. Differences between the odour profiles of lambs born in 2007 and in 2009 were thus anticipated. Fortunately five of the experimental ewes had lambs in 2007 as well as in 2009, so that some idea of the qualitative and quantitative magnitude of these differences could be obtained during the 2009 lambing season. Although practically all the qualitative work was done on wool from lambs born in 2007, it was evident that the quantitative data obtained in 2007 could not be employed in the bioassays envisaged for 2009. Synthetic test mixtures for bioassays during the lambing season of 2009 were thus formulated according to analytical data obtained from lambs born in 2009. Compounds that were identified just prior to, or during the 2009 lambing season could not be purchased or synthesised in time for inclusion in test mixtures. However, in 2010 it should be possible to formulate test mixtures reflecting all the analytical information that can be extracted from SEP-GC-MS analyses of the cranial wool of specific lambs for bioassays of the type discussed here.





a. Fitting a lamb with a diaper and with thin, stretchable plastic film.



b. Fitting a lamb with a hoodless fleece jacket.



c. Ewe investigating an alien lamb fitted with a hooded fleece jacket.



d. Spraying the fleece jacket of an alien lamb with a mixture of synthetic compounds.



e. Ewe investigating alien lamb fitted with a jacket made from brown paper.



f. Alien lamb accepted at the udder.

## Plate 5.1



## References

Alexander, G., Stevens, D. and Bradley, L. R., “Fostering in sheep: experiences with the use of neatsfoot oil”, *Austr. J. Exp. Agric.* **27**: 771–778 (1987).

Alexander, G., Stevens, D. and Bradley, L. R., “Fostering in sheep: an exploratory comparison of several approaches”, *Austr. J. Exp. Agric.* **29**: 509–512 (1989).

Price, E. O., Dunn, G. C., Talbot, J. A. and Dally, M. R., “Fostering lambs by odor transfer: the substitution experiment”, *J. Anim. Sci.*, **59**: 301–307 (1984).

## CHAPTER 6

### CONCLUDING REMARKS AND OUTLOOK

Several studies of the behaviour of ewes during the short period around the birth of their lambs have shown that a ewe uses the odour of her lamb as the cue to distinguish her lamb from other lambs. It was accepted that the results of these studies are above suspicion and that there was no other acceptable interpretation of the results of these studies. The present research was based on the hypothesis that a ewe can only distinguish her lamb from other lambs if its odour differs from those of other lambs and, furthermore, that the odours of the twin lambs are identical or at least more similar than those of any randomly selected non-twin pair of lambs.

The research carried out for the purpose of the dissertation required the availability of experimental animals and the technology with which the odour of lambs could be reproducibly captured and analysed. Döhne Merino ewes were the sheep race of choice for this study because they are known to bear a very high percentage of twins. Stellenbosch University kindly made a small flock of these ewes available for the use in the study. The SEP is a simple but at the same time very sensitive device developed in the Laboratory for Ecological Chemistry for the extraction of odorants from the headspace gas or plant material, and was used in conjunction with GC-MS to analyse VOCs that were presumed to be present on the wool of lambs as constituents of the putative semiochemical cue used in kin recognition.

Assuming that the identification cue is produced by the lamb, the question arose as to whether the cue could possibly be a maternal label, *i.e.* a chemical code in the form of a mixture of compounds in a specific quantitative ratio imprinted on the lamb by the ewe. It was thought that such a maternal label could originate from traces of amniotic fluid left on the lamb at birth. Close observation of the behaviour of a ewe trying to identify her lamb, revealed that the ewe monitors the odour from any part of the body of the lamb closest to her. Wool was collected for analysis from the heads of the experimental lambs because cranial

---

---

wool was less likely to be contaminated by urine, faeces or other contaminants than wool from other parts of the lambs' bodies.

The VOCs of the wool were extracted by SEP from the headspace gas of the wool samples and analysed by GC and GC-MS. Results revealed that, as hypothesised, the volatile organic fractions of the wool samples harvested from twin lambs are practically identical, both qualitatively and quantitatively, but differ from those of other lambs in the flock. This finding was confirmed by rigorous statistical analysis of the relevant quantitative data. A total of 133 VOCs were identified in the wool collected from 16 twins. About 50% of these compounds were also detected in small quantities in the amniotic fluid samples. This led to the conclusion that the identification of a lamb by its mother is facilitated by a maternal label transferred at birth from the amniotic fluid to the lamb. However, the odour transfer could just as well be in the opposite direction: the organic volatiles could be produced by skin glands on the body of the lambs already before birth and released into the amniotic fluid. The relatively small differences in the volatile organic content of the amniotic fluid in which twin lambs are born, could be then be explained in terms of the difference in the solubilities of the different organic compounds belonging to different compound classes in amniotic fluids having somewhat different viscosities and presumably also different protein concentrations. Protein residues left on the body of a lamb could nevertheless play a role in the semiochemical communication between lamb and ewe.

The change in the odour profiles of selected lambs was studied over a seven-day period. It was found that changes in the odour progress in a similar manner in all the lambs born during the lambing season of 2007. By making use of multivariate analysis, it was found that not only did the odour profiles change, they also became increasingly similar over time from day 1 to day 7 after birth. For as long as a ewe makes use of olfactory recognition as her main form of identification of her lamb her olfactory system has to continuously adjust to the change of the odour of the lamb and she has to continue learning the odour of her lamb.

---

---

An investigation into the possibility that the amniotic fluid undergoes a process of change or degradation during the birth process revealed that the compositions of the organic volatile fractions of the amniotic fluids of first-born and second-born lambs differ. This result put paid to the idea that the unique odour profile of the lambs could originate in the amniotic fluid, which at an earlier stage seemed to be a logical conclusion based on the observation that all the constituents identified in the amniotic fluid were also present in the wool.

The role of the residual proteins in the semiochemical communication of sheep was investigated. Five proteins, namely, fibronectin, glycogen phosphorylase (liver form), serum albumin precursor, fetuin precursor and immunoglobulin lambda light (chain constant, region segment 1) were identified in the amniotic fluid samples collected from the bodies of newborn lambs, and three proteins, namely, the serum albumin precursor, foetal haemoglobin (beta chain) and immunoglobulin lambda chain (C region) were identified as wool-associated proteins. The affinity of serum albumin for long-chain fatty acids and cholesterol is known, and the identification of three long-chain carboxylic acids and two steroids as ligands of serum albumin was thus not unexpected. Because the wool-associated proteins were denatured on removal from the wool fibres, attempts to determine the wool-associated proteins' ligand-binding properties were unsuccessful. The present research has thus not succeeded in revealing whether the wool-associated proteins could potentially play an auxiliary role in the dissemination of the semiochemicals involved in the communication between lamb and ewe.

A successful series of behavioural bioassays would have been the keystone in this project. Unfortunately the programme of bioassays had to be carried out during the 2009 lambing season that in 2008, had already been planned to last only three weeks. During these three weeks quantitative analyses had to be done almost every day in order to prepare test mixtures corresponding with the wool volatile composition of the lamb that was to be replaced by an alien lamb the next day. All bioassays were carried out knowing that the mothering instinct is not developed equally strongly in all ewes, and that it was therefore possible that one ewe might accept an experimental lamb as her own because her mothering

---

---

instinct is not particularly strong, whereas another ewe might reject a similarly treated experimental lamb outright. It has to be pointed out that strong mothering instinct is another criterion that has been used in the Mariendahl breeding programme for many years, which made it even more difficult to induce ewes to accept alien lambs.

Several factors could have contributed to the disappointing results of bioassays and will have to be taken into consideration in the bioassays to be carried out during the lambing season in 2010.

- It is possible, although unlikely, that the compounds identified as wool volatile constituents are not essential components of the recognition pheromone, that other compounds carry the semiochemical message between lamb and ewe, and that the observed intra-twin similarities and inter-twin differences in the composition of the odour of lambs are merely a reflection of genetic trends.
- It was mentioned that some of the volatile wool constituents were still unidentified by the time the bioassays were carried out and it is thus possible that some of these compounds, although present in very low concentrations, could play a key role in the communication process by, for example, conveying the message “this is a lamb and not a kid” so that the rest of the semiochemical information becomes irrelevant if this part of the message is lacking. It is possible that such compounds were not detected because they are present in the wool volatile fraction in concentrations below the detection threshold of the analytical techniques that were employed.
- It is also possible that the recognition signal is conveyed or modulated by higher-boiling material that fell outside the scope of this study. With this in mind, a mixture of synthetic compounds was bioassayed in combination with purified lanolin that was used to compensate for the absence of high-boiling material in synthetic test mixtures. This experiment also produced negative results.

- 
- 
- It was probably unrealistic to expect a synthetically formulated pheromone to be capable of overriding all other cues that ewes could conceivably bring into play in the identification of their lambs. It is not known over which distances ewes can hear the bleating of their lambs and possibly the experimental ewes' lambs should have been kept in a soundproof container during bioassays. More effective odour barriers could have been devised and the legs, ears, etc. of the experimental lambs should perhaps have been fully covered. However, this factor was probably not of overriding importance because ewes readily accepted alien lambs dressed in jackets that had been worn by their own lambs.
  - Visual cues could be of critical importance when a ewe is not convinced that the odour of the alien lamb perfectly matches that of her own lamb. It must be taken into consideration that the ewes that accepted alien lambs dressed in the jackets previously worn by their own lambs could have become used to their lambs dressed in jackets. Ewes should have been conditioned to the presence of their twins dressed in jackets for a few days before bioassays were carried out with alien lambs dressed in jackets. It was probably totally unrealistic to expect a ewe to accept an alien lamb in a jacket while the other undressed twin was still present in the pen for her to use as (an) odour reference. This conclusion is supported by the conditions under which the one successful experiment was carried out at the end of the lambing season and in which an alien lamb wearing a treated jacket was accepted at the udder. The lambs of this ewe had been wearing jackets for 72 h as part of another experiment. This probably conditioned her to the presence of lambs wearing jackets and this might have facilitated the acceptance of the alien lamb. An alien lamb fitted with a jacket that was treated with a pentane extract of the wool of this ewe's lamb was accepted by the ewe and allowed to suckle without any hesitation. The ewe, accompanied by the alien lamb, was then reunited with her own twin lambs in the outside arena. The ewe sniffed at all three lambs and accepted all of them at the udder.

These considerations will be taken into consideration and implemented as far as possible during the 2010 lambing season. The fostering of alien lambs using synthetic compounds is

still seen as an achievable goal, but ingenious solutions will have to be found to address problems associated with the uniqueness of the odour of individual lambs and the constantly changing quantitative composition of the pheromone.

To the best of my knowledge, the results reported here represent the first information on volatile organic compounds that could possibly be components of a ewe-lamb recognition pheromone.