Composition and contents of classes of neutral lipids in non-starch and starch lipid extracts of flour, sourdough and *broa* (Portuguese sourdough bread)

University of Helsinki
Department of Applied Chemistry and Microbiology
General Chemistry

João M. Rocha[1,2], Paavo J. Kalo[2] & F. Xavier Malcata[1]

[1]Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Dr. António Bernardino de Almeida, P-4200-072 Porto, Portugal
[2]Department of Applied Chemistry and Microbiology, General Chemistry, P. O. Box 27 (Latokartanonkaari 11), 00014 University of Helsinki, Finland

OBJECTIVES

Broa is traditional Portuguese bread. Milled freeze-dried samples of maize and rye flours, sourdough and *broa* were extracted consecutively so as to yield non-starch (*NSL*) and starch (*SL*) lipids. Lipid extracts were washed according to the *Blight & Dyer* procedure. *NSL* and *SL* neutral lipids (*NL*) were fractionated by flash chromatography-grade using the silica gel, open column methodology. Stepwise elution was employed. The fractions were further transesterified or esterified to fatty acid methyl esters, and then analysed by gas chromatography. The method of internal standardisation was used in the quantification of lipids, and principal component analysis was employed to reveal differences in the lipid compositions.

In terms of total lipids (*NSL+SL* extracts), the sterol ester (*SE*) content was very similar in the flour (mixture of maize + rye), sourdough and *broa*. The total content of triacylglycerols (*TAG*) decreased during sourdough fermentation; those of di- and monoacylglycerols (*DAG, MAG*) increased, whereas that of free fatty acids (*FFA*) remained at the same level. The fatty acid (*FA*) composition of the *SE* class differed clearly from that of *TAG*, *DAG*, *MAG* and *FFA* in flours, sourdough and *broa*. The *FA* compositions of *DAGs* resembled those of *TAGs* in both *NSL* and *SL* extracts, in sourdough as well as in *broa*. The total neutral lipid content was 29.4, 25.8 and 26.1 mgtotalNL/Kgsample in flour mixture, sourdough and *broa*, respectively.

The high nutritional value of *broa* lipids is apparent in the high proportions of *SE* (4.0%) and *DAG* (9.4%), together with the high proportion of linoleic acid (*ca.* 50% in *SE*, *TAG*, *FFA* and *DAG*, in both extracts).

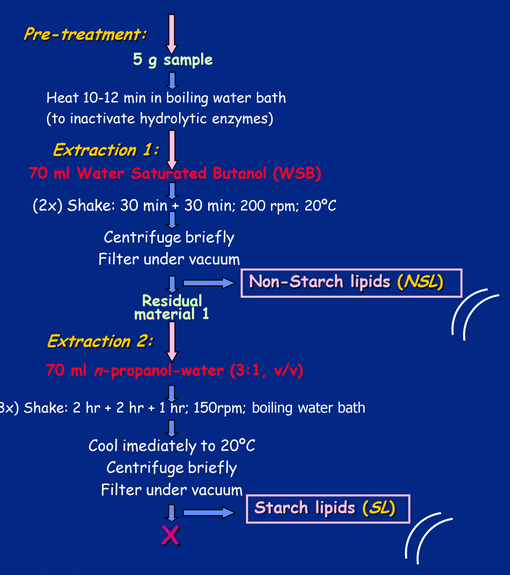
EXPERIMENTAL METHODS

MATERIALS

Source: Farmer from *Cabeceiras de Basto*

Type of samples:

- (*M*) - Maize flour
- (*R*) - Rye flour
- (*S*) - Sourdough
- (*B*) - *Broa* (bread)



Purification procedure:

Breaking lipoprotein associations:

Lipid extract + Non-lipid material
→ Evaporate solvent to driness
→ Residue taken up in a small volume of: $CHCl_3$:MeOH (2:1), ***FL*** & ***BL***; $CHCl_3$:MeOH (1:1), ***SL***
→ Add 10 drops H_2O
→ Evaporate to driness

Blight & Dyer (1959) method:

→ + $CHCl_3$, Mix briefly
→ + MeOH, Mix briefly
→ + H_2O, Mix vigorously
→ (final ratio 1:1:0.9)
→ Centrifuge briefly → Upper phase: **Non-Lipid material** (Suction under vacuum)
→ **Bottom phase: Lipid material**

Flash column chromatography fractionation:

A 5.4 g silica gel 60 (0.040-0.063mm particle size) chromatographic column was prepared in a 15 ml Isolute™ filtration column tube with micropore filters. Before adding the sample, the column was solvated and equilibrated with 50 ml hexane (Flow 4.5-5ml/min).

A precise amount of sample was added (from a volumetric flask) and an exact amount of primary set of internal standards were added: CE 17:0 + TAG 51:0 + FFA 19:0 + DAG 38:0 + Cholesterol + MAG 19:0. The solvent was evaporated and the mixture redissolved in CHCl3:MeOH (2:1 or 1:1) to final volume of 400 µl.

Stepwise elution scheme used for fractionation:

Elution solvent mixture	V (ml)	Lipid fraction	2nd ISTD
22% Dichloromethane/Hexane	60	Sterol esters	CE 13:0
65% Dichloromethane/Hexane	40	Triacylglycerols	TAG 57:0
1% Acetic acid/Hexane + 4% Dichloromethane/Hexane	40 100	Free fatty acids	FFA 17:0
3% Acetone/Dichloromethane	40	Sterols + Diacylglycerols	Cholestanol + DAG34:0
40% Acetone/Dichloromethane	40	Monoacylglycerols	MAG 17:0

→ **Trans/ Esterification**

Esterification and Transesterification procedures:

Basic transesterification of *TAG, DAG and MAG*:
Dried *TAG/ DAG/ MAG*
→ Dissolve in dried MTBE
→ 2M NaOMe/MeOH
→ Stand: 30 min reaction; Room temperature
→ GC

Acidic esterification of *FFA*:
Dried *FFA*
→ Dissolve in 5%HCl/MeOH
→ Stand: 50°C; 2hr
→ Extract with Hexane
→ Wash and drying steps:
- 5% NaCl in water
- 2% Sodium hydrogen carbonate
- Anhydrous sodium sulphate

→ GC

Basic and acidic transesterification of *SE*:
Basic: Dried *SE*
→ Dissolve in dried MTBE
→ 2M NaOMe/MeOH
→ Stand: 40 min reaction; Room temperature
Acidic
→ Dissolve in 5% HCl/MeOH
→ Stand: 50°C; 2hr
→ Extract with Hexane
→ Wash and dry
→ GC

Gas chromatography conditions

- On-column injector at 50°C and 120 kPa
- Detector at 260°C
- Carrier gas: helium; v = 35.9cm/s
- Column: 30m x 0.25mm I.D. H-P Innowax™ fused-silica capillary coated with 0.25µm film thickness
- Temperature program:

t (min)	0	3	14	15	37.5	49.5
T (ºC)	50	50	160	160	250	250

RESULTS and DISCUSSION

Lipids extracts:

Table I - Content (in mg of fraction/kg of sample) for each lipid extract and for total lipid extracts (*i.e. NSL+SL*)

Fraction	NSL	SL	TOTAL
	mg/Kg$_{flour}$		
SE	0,94	0,06	**1,01**
TAG	23,28	0,49	**23,77**
FFA	2,15	0,79	**2,94**
DAG	1,17	0,29	**1,46**
MAG	0,13	0,04	**0,17**
TOTAL			***29,35***
	mg/Kg$_{sourdough}$		
SE	0,92	0,16	**1,07**
TAG	17,78	1,00	**18,79**
FFA	1,80	0,97	**2,77**
DAG	2,47	0,32	**2,79**
MAG	0,30	0,08	**0,38**
TOTAL			***25,81***
	mg/Kg$_{broa}$		
SE	0,88	0,16	**1,03**
TAG	17,71	1,47	**19,18**
FFA	1,68	1,41	**3,09**
DAG	1,90	0,54	**2,44**
MAG	0,20	0,14	**0,33**
TOTAL			***26,08***

✓ Evolution along the fermentation and baking processes, according ***Table I*** (* - light effect):

	SE	TAG	FFA	DAG	MAG
Fermentation	↑*	↓	↓*	↑	↑
Baking	↓	≅	↑*	↓	↓

✓ In total lipids (*i.e. NSL+SL* extracts), *SE* content was very similar in flour (mixture of maize + rye), sourdough and *broa*.

✓ The total content of *TAG* decreased under the hydrolytic conditions prevailing throughout sourdough fermentation, so those of *DAG* and *MAG* increased, whereas that of *FFA* remained at the same level.

✓ *SE*, *DAG* and *MAG* contents decreased during the baking process, owing to the hydrolyses in the very beginning of the process.

✓ *TAG* was the main class in the *NSL* and *SL* fractions, except in the *SL* fraction of flours for which the *FFA* content is higher.

✓ The total neutral lipid content was 29.4, 25.8 and 26.1 $mg_{totalNL}/kg_{sample}$ in flour, sourdough and bread, respectively.

✓ As expected, the total amount of neutral lipids in *NSL* extract is much higher than in *SL* extract.

Non-Starch Lipids (NSL):

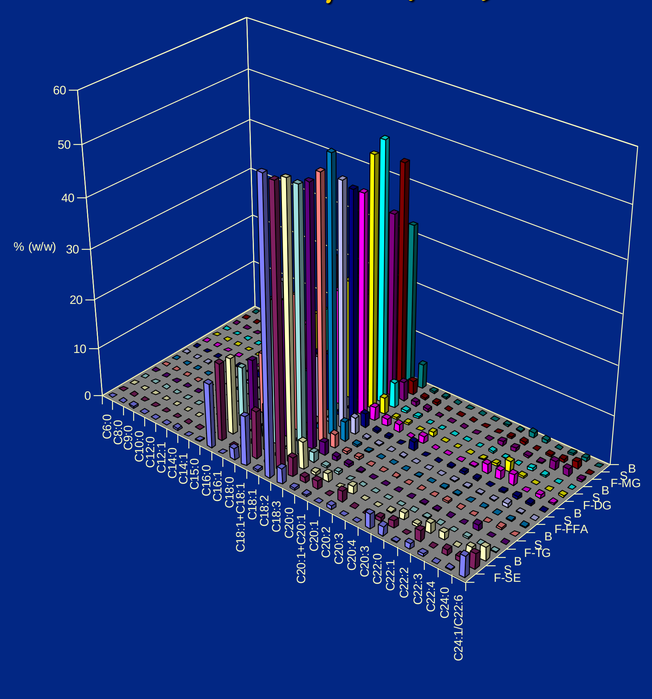
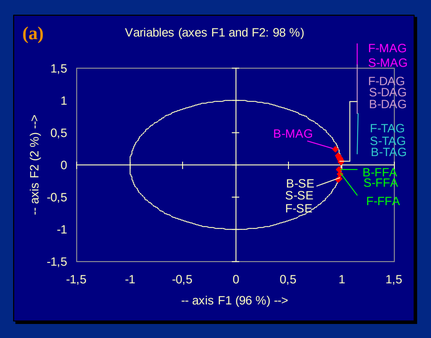


Figure 1 – *FA* composition in ***NSL*** fractions.



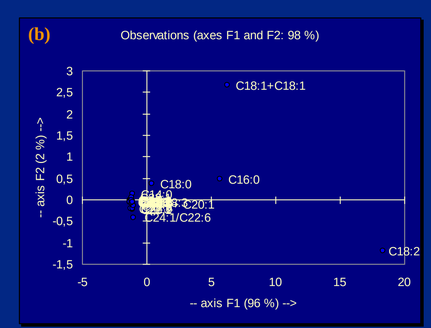


Figure 2 – Loading plot (**a**) and observation plot (**b**) on axes 1 and 2, for ***NSL***.

✓ Major *FA* in *NSL* (see ***Figure 1***) were: C16:0; C18:0; C18:1+C18:1 (methyl oleate + methyl petroselinate or methyl vaccenate); C18:2 (methyl linoleate); and C18:3 (methyl linolenate).

✓ ***Figures 1*** and ***2*** revealed that *FA* composition differs according to the fractions, which can be observed from the clusters in the correlation circles plotted on axes 1 and 2 (see ***Figure 2***). Those variables from each cluster (with high positive or negative correlations with which other) do not allow a good distinction for the objects studied, which means that the *FA* composition is not significantly different.

✓ *PC1* explains 96% of the variance in the initial matrix, whereas *PC2* explains 2%, so *PC1* and *PC2* together describe 98% (cumulative percentage) of the total variance: it was possible to reduce the 15 initial variables to only 2, with a marginal loss of information.

✓ *PCA* showed, when comparing the *NSL* extract in flours, sourdough and *broa*, that:
- C16:0 , C18:0, C18:1+C18:1 (methyl oleate + methyl petroselinate or methyl vaccenate), C18:2 (methyl linoleate) and C24:1+C22:6 (methyl nervonate + methyl docosahexaenoate) contents are the data that more strongly contribute for *PC2*, and
- *F-NSL-SE*, *S-NSL-SE*, *B-NSL-SE* and *B-NSL-MAG* are the variables with stronger positive contribution for *PC2*.

- When simultaneously comparing *SE* with the other classes, in *F*, *S* and *B*, the *PCA* indicated that the *NSL-SE* have consistently:
  = the highest values for C18:2 (methyl linoleate) and C24:1+C22:6 (methyl nervonate + methyl docosahexaenoate), as well as for C20:3 (methyl *11-14-17* eicosatrienoate), C22:0 and C22:2 (methyl *13-16* docosatrienoate) contents, and
  = the lowest values for C18:1+C18:1 (methyl oleate + methyl petroselinate or methyl vaccenate) contents, and the lowest C18:0 content in *S* and *B-NSL-SE* ;

- the *B-NSL-MAG* yields a distinct situation, because of the lowest values of C18:2 (methyl linoleate) content and one of the highest contents of C16:0 and C18:0.

✓ Other clusters were observed:
- All samples from *NSL-FFA* were:
  = characterized by the highest or one of the highest contents of C16:0 and C18:1 (methyl petroselinate or methyl vaccenate); and
  = the lowest or one of the lowest contents of C20:0 and C20:1+C20:1 (methyl *5*-eicosenoate + methyl *8*-eicosenoate);

- With regard to *NSL* from *TAG*, *DAG* and *MAG*:
  = except for *B-NSL-MAG*, all fractions of *TAG*, *DAG* and *MAG* from *NSL* are relatively close to one another;
  = *NSL-TAG* have the lowest content of C16:0, and *DAG-NSL* yield low values as well;
  = *NSL-TAG* have the highest content of C18:1+C18:1 (methyl oleate + methyl petroselinate or methyl vaccenate), and *NSL-DAG* exhibit high values as well; and
  = *NSL-TAG* have the lowest C18:3 (methyl linolenate) and C22:0 contents, and *MAG-NSL* have one of the highest contents of C22:1 (methyl erucate), C22:2 (methyl *13-16* docosadienoate) and C22:3 (methyl *13-16-19* docosatrienoate).

Starch Lipids (SL):

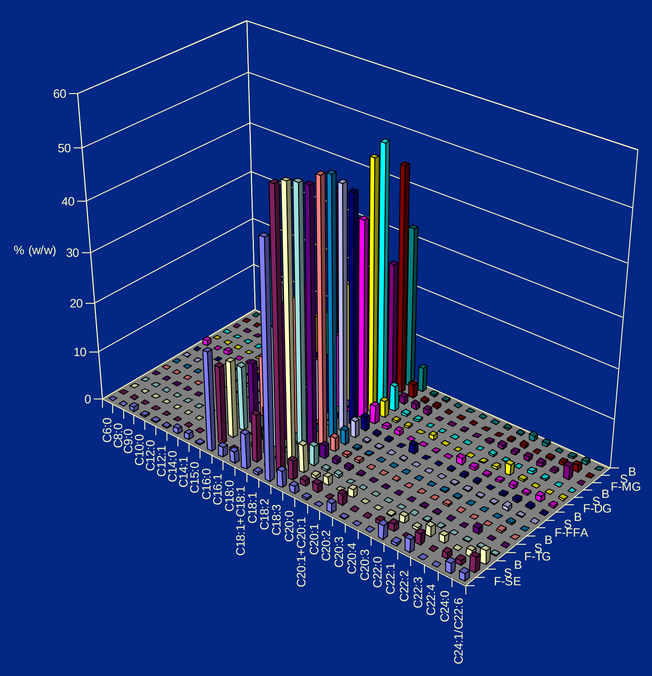
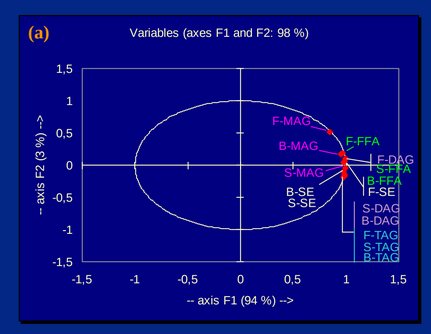


Figure 3 – *FA* composition in ***SL*** fractions.



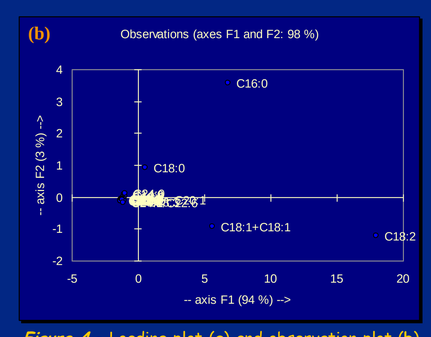


Figure 4 – Loading plot (**a**) and observation plot (**b**) on axes 1 and 2, for ***SL***.

✓ *Figure 3* shows that the major *FA* in *SL* are: C16:0; C18:0; C18:1+C18:1 (methyl oleate + methyl petroselinate or methyl vaccenate); C18:2 (methyl linoleate); C18:3 (methyl linolenate); C20:0; and C20:1+C20:1 (methyl 5-eicosenoate + methyl 8-eicosenoate).

✓ The results by *PCA* for *SL* (first two *PCs* describe together 97.8% of the total variance in the initial matrix) revealed that C16:0, C18:0, C18:1+C18:1 (methyl oleate + methyl petroselinate or methyl vaccenate) and C18:2 (methyl linoleate) account for the highest contribution to *PC2* (see *Figure 4*) , which means a further contribution to discriminate between the fractions.

✓ The sample with strongest contribution for *PC2* is *F-SL-MAG*, because of its singular profile, with high contents of C16:0, C18:0 and low content of C18:2 (methyl linoleate).

✓Some clusters could be defined, although the distinction is not as clear as in *NSL*:
- with regard to *SL-TAG*,
  = they exhibit the highest content of C18:1+C18:1 (methyl oleate + methyl petroselinate or methyl vaccenate), and one of the highest contents of C18:2 (methyl linoleate), and
  = the lowest content of C16:0 and C22:0;

- *SL-TAG* and *SL-DAG* resemble together,
  = with one of the highest levels of C18:1+C18:1 (methyl oleate + methyl petroselinate or methyl vaccenate), and
  = one of the lowest contents of C16:0;

- in what concerns *SE* from *S* and *B*,
  = one found the highest content of C18:2 (methyl linoleate) and C20:1+C20:1 (methyl *5*-eicosenoate + methyl *8*-eicosenoate), and
  = the lowest C18:0 content;

- All samples of *SL-SE* exhibited
  = the lowest content of C18:1+C18:1 (methyl oleate + methyl petroselinate or methyl vaccenate), and
  = the highest content of C20:2 (methyl *11-14* eicosadienoate), C22:0, C22:2 (methyl *13-16* docosadienoate) and C24:1+C22:6 (methyl nervonate + methyl docosahexaenoate).

CONCLUSIONS

❑ The PCA (not shown) carried out on the experimental results showed also that, despite the high correlation factors between samples, the type of product (*i.e.* flours, sourdough and bread) can generally be distinguished on the basis of their *FA* profile:
- In the fractions of *SE*, *TAG* and *DAG* from the *NSL* extract, flours present the highest C18:0 and the lowest C16:0 contents;
- In the fractions of *SE*, *TAG* and *DAG* from the *SL* extract, flours present the highest C16:0 and C18:1+C18:1 (methyl oleate + methyl petroselinate or methyl vaccenate) contents;
- Unlike the *MAG* fraction, the *FA* composition of *S* and *B* resembles are close to each other.

❑ Unlike *TAG* during the fermentation process, the content of all the other lipid classes but *FFA* increases.

❑ During the baking process, there is a loss of *SE*, *DAG* and *MAG* contents.

❑ The dominant lipid classes differ between *TAG* and *FFA*, according to the lipid extract and the sample. Despite the fact that *FA* compositions differ depending on the fractions, C16:0, C18:0, C18:1+C18:1 (methyl oleate + methyl petroselinate or methyl vaccenate) and C18:2 (methyl linoleate) contents are the *FA* present in major amounts, and which more strongly contribute for the distinction between lipid fractions.

❑ The *FA* composition of the *SE* class differs clearly from that of *TAG*, *DAG*, *MAG* and *FFA* classes in flours, sourdough and *broa*. This distinct profile is shown clearly by the cluster formed by *F*, *S* and *B*, in the *NSL-SE* and *SL-SE* fractions, although the *FA* profile is more similar for *S* and *B* than with *F*.

❑ The *FA* composition of *DG* resemble that of *TG* in both *NSL* and *SL* extracts, in sourdough and *broa*.

❑The high nutritional value of *broa* lipids is apparent via the high proportions of *SE* (4.0%) and *DG* (9.4%), together with the high proportion of linoleic acid (*ca.* 50% in *SE*, *TG*, *FFA* and *DG*, in both extracts).

ABBREVIATIONS

Maize flour - *M*
Rye flour - *R*
Mixture of maize and rye flours - *F*
Sourdough - *S*
Broa (bread) - *B*

Non-starch lipids - *NSL*
Starch lipids - *SL*

Neutral lipids - *NL*
Fatty acid - *FA*
Sterol ester - *SE*
Triacylglycerols - *TAG*
Fatty acids - *FFA*
Diacylglycerols - *DAG*
Monoacylglycerols - *MAG*

Chloroform - *CHCl3*
Methanol - *MeOH*
Water - *H2O*
HCl - *Hydrogen chloride*
Methyl-tert-butyl ether - *MTBE*
NaOMe - *Sodium methoxide in methanol*
NaCL - *Sodium chloride*

Principal Component Analysis - *PCA*
Principal Component - *PC*

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