

TRUEFOOD

Traditional United Europe Food

Contract no. FOOD-CT-2006-016264

Instrument: Integrated Project

Thematic Priority: Food Quality and Safety (# 5)

D2B.5.4

Interim report on the use of NMR technique for assessment migration from packaging materials

Due date of deliverable: October 2007
Actual submission date : November 2007

Start date of project: 1 May 2006

Duration: 48 months

Organization name of lead contractor for this deliverable: ENEA

Revision final

Project co-funded by the European Commission within the Sixth Framework Programme (2002-2006)		
Dissemination Level		
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Interim report on the use of NMR technique for assessment migration from packaging materials.

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Summary:

The tests for the measurement of migrating substances from packaging systems to food are generally accomplished by using simulant substances in order avoid the interference of the complex chemical structure of the food with the analytical process. One of the used simulant for fatty foods is olive oil which however may produce interferences when NMR is used for the detection of contaminants and sometimes in chromatography. Several alternative molecules to olive oil have been synthesized and tested in terms of ability to extract contaminants from the polymer films and in terms of analytical characteristics. One of the selected new simulants, namely dibutyl sebacate is then used in one successive migration experiment.

NMR is used to study specific and overall specific migration from nisin based active packaging films. The migrating substance profile from nisin based films was obtained by NMR spectra from four type of simulants in two conditions of temperature and exposition time.

Preliminary studies on the determination of specific migration on food were accomplished on provolone Italian cheese. Actually, NMR spectra of provolone cheese are acquired and nisin resonances identified in the aqueous extract of the cheese.

Part I: Alternative simulant to olive oil for migration tests

Introduction

The tests for the measurement of migrating substances from packaging systems to food are generally accomplished by using simulant substances in order to avoid the interference of the complex chemical structure of the food with the analytical process. Different simulants are actually used depending on the polarity of the migrating substances to be extracted from the packaging material. Water, ethanol, iso-octane and rectified olive oil are the most common used simulants. However, olive oil can show interferences in NMR (the spectra is complex due mainly to the double bonds of triglycerides) and sometimes in chromatography. Moreover ethanol 95% and isooctane, the food simulants used instead of olive oil, do not give the same kind of interactions with molecules contained in the packaging and the migration could be quite different from the use of olive oil. Therefore it seems essential to develop a simulant closer to olive oil in terms of structure and interactions.

This simulant can be based on the simulant HB 307, a mixture of triglycerides (8 to 12 carbons) but it is all the more important that the simulant contains ester and long alkyl chains to show the same kind of interactions than olive oil.

First, to bypass interferences from compounds present in olive oil, a purification of this simulant by liquid/liquid extraction will also be performed.

We then try to synthesise by a very simple reaction (esterification) different molecules which have similar interactions than olive oil. Thus, anyone can produce the new simulant in a very simple manner with basic laboratory equipments. Molecules which are synthesised with the best yield and purity will be produced in larger scale quantities.

The second part of this study will be to assess the migration power of the new simulants and compare them to olive oil's. NMR spectra will be also recorded to ensure there are no interferences.

Screening of alternative molecules

The molecule synthesised must fulfil some criteria :

- Presence of at least one **ester**
- **Long alkyl chains**
- The simulant must have a **NMR** spectra with a **large region free of signals** which means few functionalities and a **simple structure**
- **Easy and safe** to make
- Have **at least the same migration power than olive oil**
- And **cheap** (less than 80-100€/kg)

The ester can come from the condensation of alcohol and acid in acidic media :

- Mono ester : fatty carboxylic acid + small chain alcohol or long chain alcohol + small carboxylic acid;
- Diester : diacid (short or long chain) + alcohol (long or short chain) or dialcohol (short or long chain) + acid (long or short chain).
- Triester (=triglyceride) : glycerol + acid (short or long chain)

The combinations of alcohol and acid that will be tested depend on the compounds available shortly in the laboratory.

Selection of the best molecules

A.1. What is olive oil?

The analysis of olive oil is performed (NF EN ISO 5509) by saponification of the triglycerides and esterification of the formed fatty acids with boron trifluoride.

Methylesters are then extracted with hexane and analysed by GC/MS.

Ester	Formula	Proportion
Methyl palmitate	$\text{CH}_3(\text{CH}_2)_{14}\text{COOMe}$	7%
Methyl linoleate	$\text{CH}_3(\text{CH}_2)_4\text{CH}=\text{CHCH}_2\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOMe}$	2%
Methyl oleate	$\text{CH}_3(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOMe}$	89%
Methyl stearate	$\text{CH}_3(\text{CH}_2)_{16}\text{COOMe}$	2%

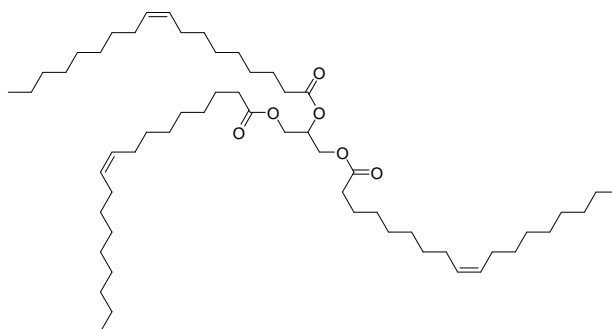


Figure 1 - Glycerol trioleate

A.2. Purification of olive oil

In order to remove the maximum of interferences such as free fatty acid, proteins, small organic molecules and so on, olive oil is submitted to liquid/liquid extractions.

Two procedures, one with basic extraction, one without, have been performed:

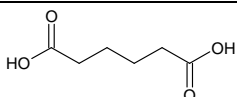
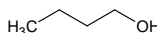
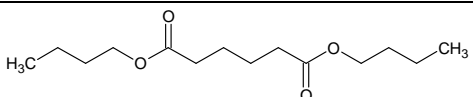
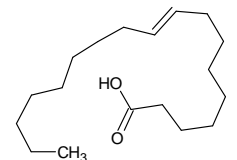
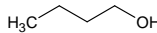
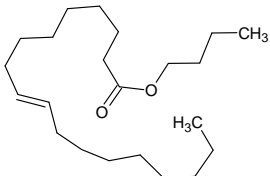
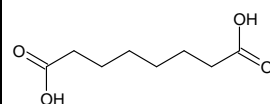
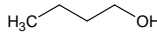
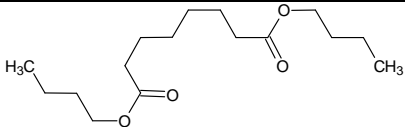
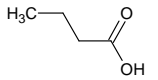
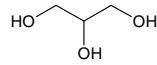
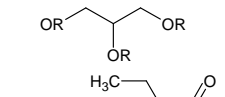
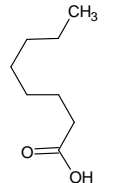
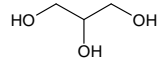
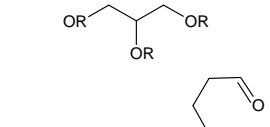
- Methanol wash; to remove small organic compounds
- Basic wash; to remove free fatty acids and acidic molecules

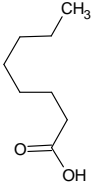
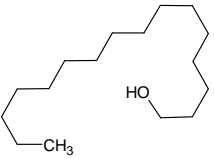
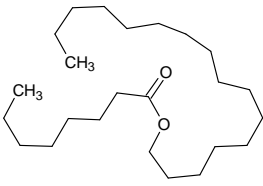
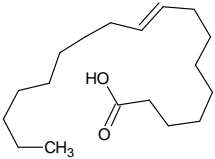
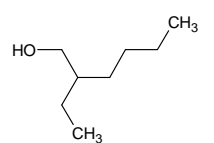
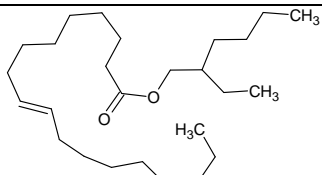
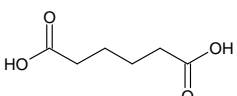
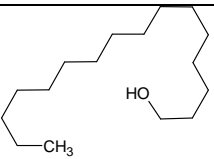
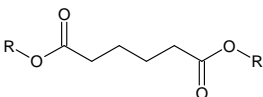
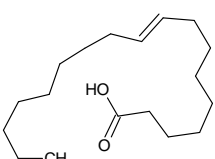
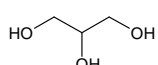
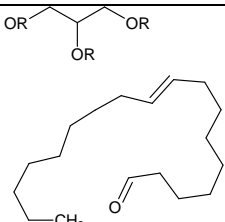
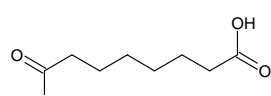
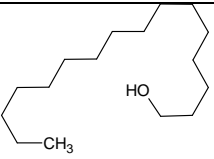
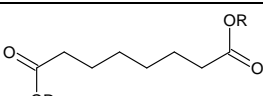
- Acid wash; to remove basic compounds (proteins)
- Sodium chloride solution wash; to remove acidity and water.

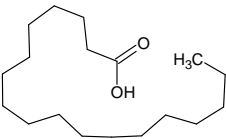
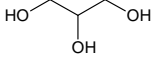
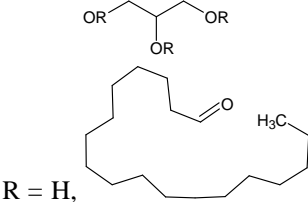
IR spectrum (ATR horizontal) of purified olive oil and raffinated olive oil are identical and NMR analysis also do not show significant differences. Indeed, glyceride oleate remains the main product and these techniques are not very sensitive to detect impurity under 5%.

Synthesis

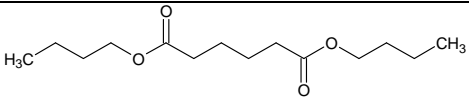
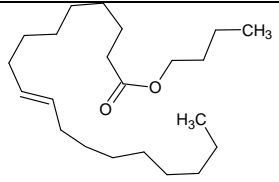
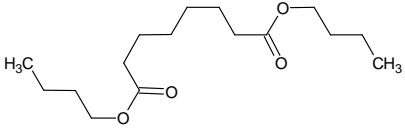
As described in the introduction, several grams of different esters have been synthesised regarding the materials available in a short delay in the laboratory:

Acid	Alcohol	Ester	Yield on the crude (Purity determined by GC/FID)	Analysis	Notes
 <p>Adipic acid</p>	 <p>n-Butanol</p>	 <p>di n-Butyl adipate</p>	<p>74% (90%) with n-butanol</p> <p>83% (88%) with n-butanol</p>	<p>GC/FID</p> <p>GC/MS</p> <p>IR</p> <p>NMR</p>	
 <p>Oleic acid</p>	 <p>n-Butanol</p>	 <p>n-Butyl oleate</p>	<p>73% (84%) with n-butanol</p> <p>82% (80%) with n-butanol</p> <p>68% (82%) with n-butanol</p>	<p>GC/FID</p> <p>GC/MS</p> <p>IR</p> <p>NMR</p>	<p>Oleic acid is only 90% pure.</p> <p>Oleic acid tends to decompose in sulphuric media.</p> <p>Product is very sensitive to heat (60-70°C).</p>
 <p>Sebacic acid</p>	 <p>n-Butanol</p>	 <p>di n-Butyl sebacate</p>	<p>95% (90%) 84% (74%) with n-butanol</p>	<p>GC/FID</p> <p>GC/MS</p> <p>IR</p> <p>NMR</p>	
 <p>Butanoic acid</p>	 <p>Glycerol</p>	 <p>R = H, Glyceryl butanoate</p>	<p>44% (72%) in mixture with the mono and diester glyceride</p>	<p>GC/FID</p> <p>GC/MS</p> <p>IR</p>	<p>A centrifugation is needed in order to separate the excess of potassium butanoate during purification.</p> <p>Butanoic acid has a very bad smell which makes it very unpleasant to manipulate.</p>
 <p>Octanoic acid</p>	 <p>Glycerol</p>	 <p>R = H, Glyceryl trioctanoate</p>	<p>128% (51%) In mixture with octanoic acid 116% (3%?) Mainly octanoic acid</p>	<p>GC/FID</p> <p>GC/MS</p> <p>IR</p> <p>NMR</p>	<p>Octanoic acid tends to decompose in sulphuric media.</p> <p>Reaction seems no to have occurred. Octanoic acid not enough reactive?</p> <p>Problems of precipitation of octanoic acid during purification</p>

Acid	Alcohol	Ester	Yield on the crude (Purity determined by GC/FID)	Analysis	Notes
 <p>Octanoic acid</p>	 <p>Palmitol</p>	 <p>Palmityl octanoate</p>	98% (87%) but solid at R.T.	GC/FID IR	Problems during purification due to the surfactant nature of potassium octanoate.
 <p>Oleic acid</p>	 <p>2-Ethyl-1-hexanol</p>	 <p>2-Ethyl-1-hexanyl oleate</p>	53% (80%)	GC/FID GC/MS IR	Oleic acid is only 90% pure. Oleic acid tends to decompose in sulphuric media.
 <p>Adipic acid</p>	 <p>Palmitol then methanol</p>	 <p>R = CH₃, C₁₆H₃₃ Di palmityl adipate, di methyl adipate or methyl, palmityl adipate</p>	-	IR	Crude is solid. No further purification
 <p>Oleic acid</p>	 <p>Glycerol</p>	 <p>R = H, Glyceryl trioleate</p>	-		Triglycerides were inseparable from starting material No further purification were performed.
 <p>Sebacic acid</p>	 <p>Palmitol then n-butanol</p>	 <p>R = C₄H₉, C₁₆H₃₃ di palmityl sebacate, di n-Butyl sebacate or n-butyl, palmityl sebacate</p>	-	GC/MS IR	The esterification is not complete and a simple purification of the crude was unsuccessful.

Acid	Alcohol	Ester	Yield on the crude (Purity determined by GC/FID)	Analysis	Notes
 <p>Stearic acid</p>	 <p>Glycerol</p>	 <p>R = H, Glycerol tristearate</p>	-		<p>Stearic acid was not soluble in the media even by increasing the temperature</p>

The esters in bold are the ones selected for a large-scale production. Indeed these esters are easy to make with good yields :

Ester	Average yield (Average purity)
 <p>di n-Butyl adipate</p>	79% (89%)
 <p>n-Butyl oleate</p>	74% (82%) with n-butanol
 <p>di n-Butyl sebacate</p>	90% (82%) with n-butanol

Unfortunately it is impossible to obtain triglycerides in a very simple manner. The reaction leads to a complex mixture which can only be purified by using preparative chromatography and/or large volumes of water and organic solvents. The procedure would no longer be cost effectively and ecologically viable.

Large scale

In order to perform migration tests at least 700-800g of each simulants must be obtained. By using the same protocol developed earlier large scale of purified olive oil and each ester are prepared.

A.3. Results

Ester	Yield (Purity)	Quantity (g)	Notes
Olive oil A	-	-	Procedure with basic extraction
Olive oil B	-	-	Procedure without basic extraction
di n-Butyl adipate	104% (79%) with n-butanol	952	
n-Butyl octanoate	90% (93%)	842	
di n-Butyl sebacate	91% (76%) with n-butanol	918	
n-Butyl oleate	95% (94%)	745	Oleic acid tends to decompose in sulphuric media.

Note : n-butyl octanoate was prepared only in large scale. Due to the shorter alkyl chain of the ester (less interaction of n-butanol with the final product) and the smaller amount used in this synthesis, n-butanol seems to be easily removed.

It takes about **a day to synthesise 2 batch of 700-800g of ester** since we are limited by the volume of the flask (2L) where the reaction occurs.

Costs

The following costs are estimated regarding the price of Aldrich or our supplier of olive oil in the needed quality. As it was said before the simulant must be as cheaper as possible.

Compounds	Mw (g/mol)	d	€/kg	€/L	€/mol
Glycerol	92	1.25	31.44	39.3	2.89
Octanoic acid	144	0.91	31.4	28.6	4.5
Butan-1-ol	74	0.81	19.8	16.0	1.5
Adipic acid	146	-	18.6	-	2.7
Sebacic acid	202	-	57.4	-	11.6
Oleic acid 90%	282	0.89	38.6	34.4	-
Olive oil	-	0.91	6.21	5.65	-
Methanol	32	0.79	-	3.8	-
Hydrochloric acid 1N	36.5	1	2.61	2.61	-
Sulfuric acid	98	1.84	7.7	14.1	-
Potassium carbonate	138	-	19.8	-	2.73
Sodium chloride	58.5	-	12.7	-	-
Anhydrous sodium sulfate	142	-	21.52	-	-

The costs are evaluated on the basis of the largest production.

Simulant	Compound	Quantity (Purity)
Olive oil A	Olive oil	680g
	Methanol	500mL
	Potassium carbonate	17.3g
	Sodium chloride	250g
	Hydrochloric acid 1N	500mL
	Product	517g 76% (-)
Olive oil B	Olive oil	550g
	Methanol	400mL
	Sodium chloride	200g
	Hydrochloric acid 1N	400mL
	Product	535g 97% (-)
di n-Butyl adipate	Adipic acid	336g
	Butanol	851g
	Sulfuric acid	135g
	Potassium carbonate	150g
	Sodium chloride	250g
	Anhydrous sodium sulphate	360g
	Product	558g 94% (79%)
n-Butyl octanoate	Octanoic acid	677g
	Butanol	639g
	Sulfuric acid	64g

Simulant	Compound	Quantity (Purity)
	Potassium carbonate	90g
	Sodium chloride	70g
	Anhydrous sodium sulphate	200g
	Product	842g 90% (93%)
di n-Butyl sebacate	Sebacic acid	424g
	Butanol	777g
	Sulfuric acid	123g
	Potassium carbonate	140g
	Sodium chloride	70g
	Anhydrous sodium sulphate	180g
	Product	602g 91% (76%)
n-Butyl oleate	Oleic acid 90%	727g
	Butanol	687g
	Sulfuric acid	68g
	Potassium carbonate	100g
	Methanol	200mL
	Anhydrous sodium sulphate	180g
	Product	745g 95% (94%)

Note : di n-Butyl sebacate is an ester available in Aldrich for 86€/kg (97% purity).

The purifications of olive oil seems to be the most efficient simulant regarding the cost of production but the purifications and especially the basic extraction lead to emulsions which are very difficult to break. Moreover it implies the use of centrifugation which is not an easy way to purify large quantities. Finally, purified olive oil do not have a large domain free of signals in NMR which will not facilitate the study of active packaging.

Every compound can be prepared with high yield. However **n-dibutyl sebacate** is commercially available with a certified purity. Since all partners can not produce this molecule at a large scale, it seems then the ester the more efficient to replace olive oil in term of cost in time. We also be sure to work with a simulant which has a constant quality.

We will study in the next part the capacity of the prepared esters to replace olive oil in term of migration.

Migration assessments

Once the substitutes have been synthesised in the convenient way, the next step is to assess the capability of the liquids to replace olive oil as food simulant. To do so, two polyethylene films which contain known additives are subjected to specific migration tests using olive oil and the prepared liquids as food simulants.

The migration values of chosen additives in the prepared liquids are compared with the ones obtained after migration in olive oil. To be qualified, olive oil substitute must have at least the same migration power than olive oil.

Migration conditions

The migrating conditions used to evaluate the products are those described in the directives 82/711/CEE, 85/572/CEE and 2002/72/CEE :

One dm² of each film is immersed in 100mL of simulant and heated at **40°C for 10 days**. For each film (2 polyethylene films) and each simulant (olive oil, 2 purified olive oils and 4 esters) the test is carried out three times.

Analytical development

Markers

To evaluate the migration performances of the simulants, **one or two additives by film are chosen to characterize the migration**. The main criteria for the selection was the ease of analysis of these markers.

Films with antimicrobial agents were not yet available at the time of this study. Thus, additives were picked from on hand films. **Chimasorb 81 and Tinuvin 326**, two UV-stabilizer, are selected for their high UV absorption and their ease of separation in HPLC/UV from the matrix and other interferences.

Their concentrations in the master mixtures are the following :

	Polyethylene 2	Polyethylene 3
Chimasorb 81	5000mg/kg	1000mg/kg
Tinuvin 326	-	1000mg/kg

During the process of extrusion, unknown amounts of these additives are degraded and we will not expect to recover the initial quantity in the film.

Extraction

The next step is to be able to extract the markers from the fatty matrix. Indeed, sample can not be injected without further treatment since fatty media is not miscible with chromatographic eluant.

The easiest way to remove an analyte from a matrix is to perform a liquid/liquid extraction. Unfortunately selected additives are **more soluble in olive oil than in water** since they can make similar apolar interactions to olive oil or aliphatic esters. The best

extraction yield was only 15%. Any other organic solvent are miscible with the ester and can not be used as extraction solvent.

Solid Phase Extraction (SPE)

Solid phase extraction (SPE) is a method for rapid sample preparation in which a solid stationary phase is typically packed into a syringe barrel and used to selectively extract target analytes prior to analysis by HPLC. The use of this technique is motivated by the fact that additives can make donor hydrogen bonds with silanol of a ungreffed silice thanks to their hydroxyl group. We hope that this interaction is strong enough to elute selectively additives and matrix.

Due to a lack of time, only ungreffed silice sorbent is tested and unhappily additives were **eluted with the matrix** (olive oil) despite several modifications of the SPE protocol.

No other sorbents are studied but antimicrobial agents are much more polar than common additives and this technique of purification will be detailed for the assessment of the active packaging.

Dilution

The last way to analyse the migration simulants is to enough dilute the fatty liquid so the sample becomes miscible with the HPLC eluant. This technique badly decreases the sensibility and the ability to detect small amounts of additive. Indeed to be miscible the sample must be **diluted by a factor 50**. An exact amount of simulant (around 100mg) is diluted in 5mL of a mixture of tetrahydrofuran and water (85:15).

Due to the high concentrations of additives in the films, we are however able to quantify additives in spite of the dilution.

HPLC quantification

The simulants are analysed by HPLC/UV (C8 column). Each migration test is injected three times to make sure of the repeatability of the analysis.

Results

First of all, blank simulants are analysed to be sure that none compound will interfere with markers.

The assessment is performed on the response of the analyte (area of the pic) in UV detection. The migration values from olive oil are then determined to calculate yield of migration for the other simulants.

Olive oil

(Area Unit)	Chimasorb 81 in Polyethylene 2			Chimasorb 81 in Polyethylene 3			Tinuvin 326 in Polyethylene 3					
	Weight sample (mg)	Inj 1	Inj 2	Inj 3	Weight sample (mg)	Inj 1	Inj 2	Inj 3	Weight sample (mg)	Inj 1	Inj 2	Inj 3
Migration test 1	99.1	104385	104718	104059	99.6	9516	8854	8572	99.6	14096	14631	15040
Migration test 2	106.3	105800	102919	108385	102	11419	12536	10652	102	16959	15136	15660
Migration test 3	102.9	107186	101579	103869	102.2	12143	11821	11115	102.2	16014	16524	16694

The results are very repeatable from one injection to another.

	Chimasorb 81 in Polyethylene 2	Chimasorb 81 in Polyethylene 3	Tinuvin 326 in Polyethylene 3
Average of the 9 injections normalized to a 100mg sample weight (AU)	102016	10589	15437

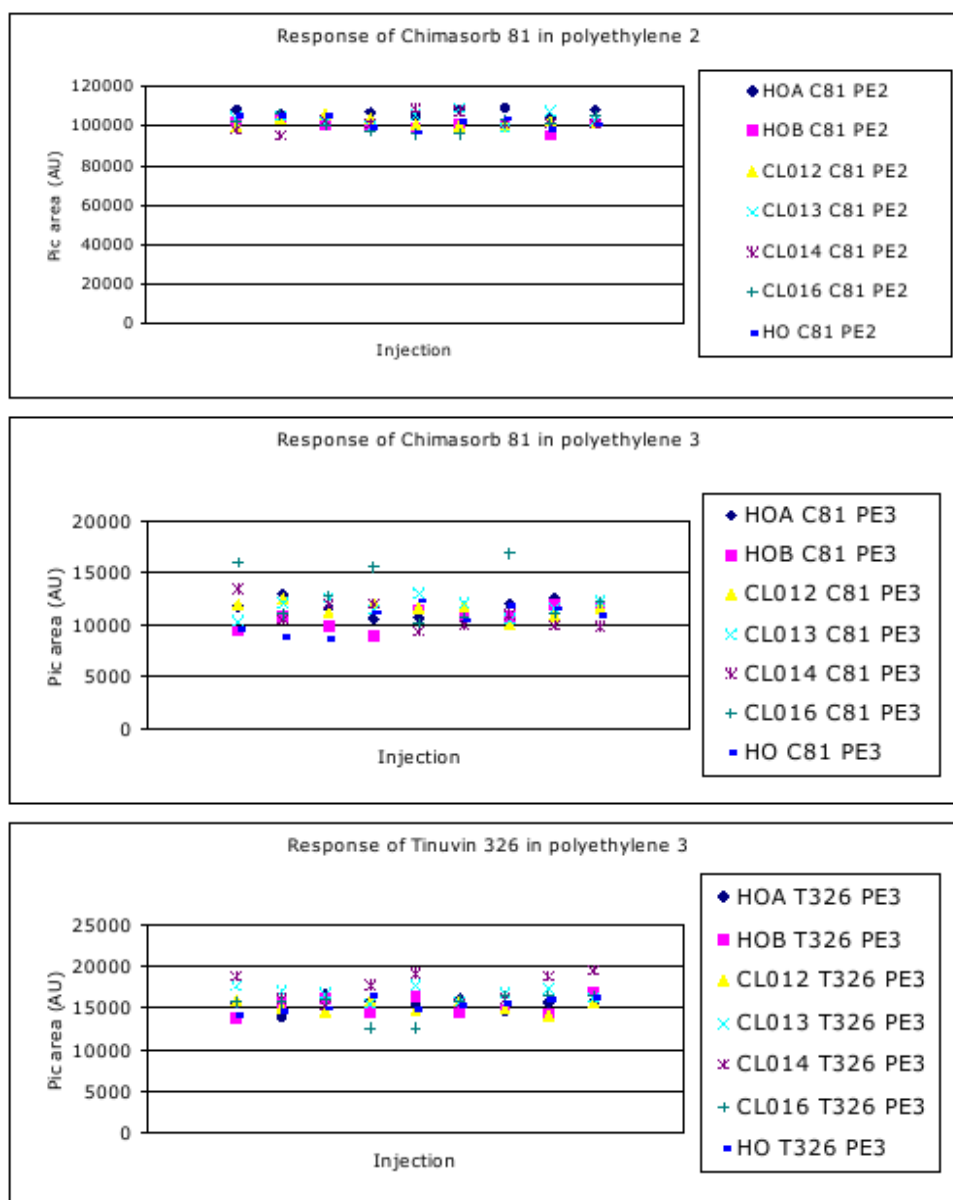
These values will be used to calculate the yield of migration from each simulant referring to olive oil.

Qualitative assessment

The following graphics show the 9 responses (3 migration tests * 3 injections) of the simulants for each couple marker/film.

The references in the graph are explained in the following table :

Reference	Simulant
HO	Olive oil
HOA	Olive oil purified by protocol A
HOB	Olive oil purified by protocol B
CL012	di n-Butyl adipate
CL013	di n-Butyl octanoate
CL014	di n-Butyl sebacate
CL016	di n-Butyl oleate

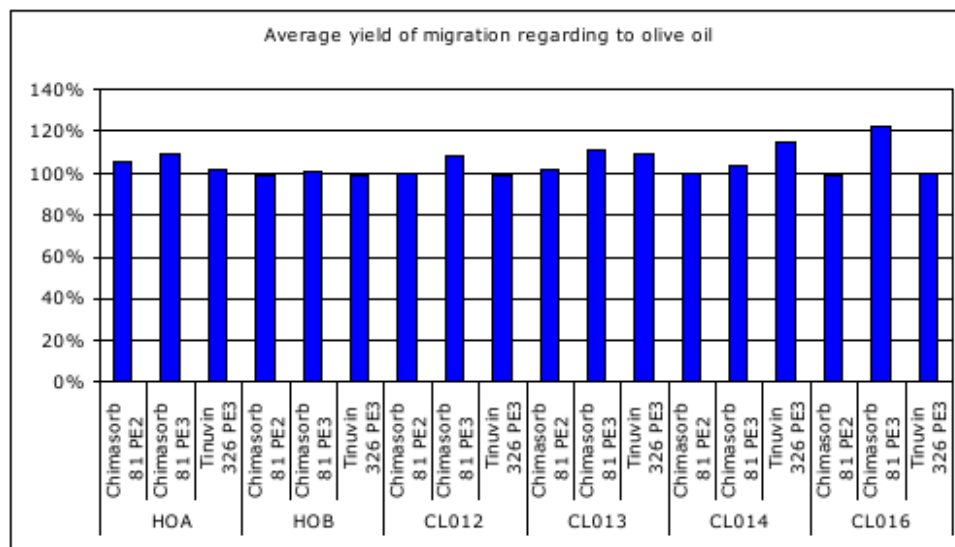


Graphically the results are consistent except for the migration of Chimasorb 81 from PE3 in di n-Butyl oleate (CL016) where the values are on average 22% higher.

A statistic study (standard deviation and relative standard deviation) has been made on the repeatability of results but it is not shown in this report. The relative standard deviations are lower than 20% and are on average 6%.

Quantitative assessment

All response values are normalized to a 100mg sample weight and an average yield of migration for each couple marker/film is calculated.



As a **migration** point of view, **all food simulants** prepared are **as efficient as olive oil**.

Conclusions

To conclude, all simulants prepared are suitable to replace olive oil. They are all easy to make in a safe way. They contain ester and long alkyl chains functionalities. What is the most important is that they all have the same migration power than olive oil and show large domain free of signals in NMR which means the simplest structure.

Due to its commercial source, **di n-butyl sebacate** is the solvent which has the more advantage for the partner. The NMR spectra has a large domain free of signals, it has the same migration power than olive oil and it can be provided in a commercial way with a constant quality.

Protocols

Since the synthesis or the purifications involve working with volatile organic solvent, the laboratory works must be done **under hood**.

Needed materials

- Separatory funnel (2L)
- Small laboratory materials
- 2L-one neck flat-bottom flask
- Adapted magnetic stirring bar
- Heating and stirring apparatus
- Heating bath (silicon oil)
- Thermometer
- Balance with 0.1g precision.
- Büchner funnel or a funnel fitted with a piece of cotton
- Rotary evaporator in which a vacuum of at least 100mmHg (131 mbar) can be applied

Purification of olive oil

The separation between the two layers can take time (sometimes 1h) especially with aqueous treatment. It furnishes quicker results to give a rotary movement to the ampoule but the more important is to be very patient.

Protocol A

In a 2L separatory funnel, 680g of olive oil are introduced. The fatty layer is extracted by 1*250mL of methanol. The lower layer (Olive oil) is then extracted by 1*250mL of a solution of potassium carbonate (0.5M)/sodium chloride (200g/L). The mixture becomes white (precipitation of free fatty acids) and is centrifuged (5000rpm for 15min). The clear upon layer (olive oil) is then extracted by 2*250mL of a solution of hydrochloric acid (1M)/sodium chloride (200g/L) and 2*250mL of a solution of sodium chloride (200g/L). Finally, the olive oil layer is extracted by 1*250mL of methanol and the lower layer is centrifuged (5000rpm for 15min) to afford 517g (76%) of purified olive oil.

Protocol B

In a 2L separatory funnel, 550g of olive oil are introduced. The fatty layer is extracted by 1*200mL of methanol. The lower layer (Olive oil) is then extracted 2*200mL of a solution of hydrochloric acid (1M)/sodium chloride (200g/L) and 3*200mL of a solution of sodium chloride (200g/L). Finally, the olive oil layer is extracted by 1*200mL of methanol and the lower layer is centrifuged (5000rpm for 15min) to afford 535g (97%) of purified olive oil.

Synthesis

Concentrated sulphuric acid is very corrosive and must be handled with gloves, goggles and a smoke. Others compounds are not harmful but must be handled with care.

di n-Butyl adipate

In a 2L-one neck flat-bottom flask equipped with a magnetic stirring bar, 336g (2.3mol) of adipic acid and 851g (11.5mol, 5eq) of n-butanol are introduced. 135g (1.38mol, 0.6eq) of concentrated sulphuric acid are added and the mixture is heated at 60°C for 3h. First of all adipic acid is not soluble (white solid) but the mixture becomes limpid with the degree of conversion of the reaction.

The mixture is allowed to cool to room temperature and excess of butanol is removed under vacuum (the temperature of evaporation must be under 60°C). 500mL of water are added. 150g of potassium carbonate are added very slowly (pH must be superior to 8). 180g of sodium chloride are added and the mixture is well stirred. The two layers are separated and the upon layer is extracted with 200mL of a saturated solution of sodium chloride (350g/L). The upon layer is dried under 180g of anhydrous sodium sulphate for 1h and filtered on Büchner funnel to afford 558g (94%) of di n-butyl adipate.

di n-Butyl octanoate

In a 2L-one neck flat-bottom flask equipped with a magnetic stirring bar, 676.8g (4.7mol, 2.18eq) of octanoic acid and 639.4g (8.64mol, 4eq) of n-butanol are introduced. 63.7g (0.65mol, 0.3eq) of concentrated sulphuric acid are added and the mixture is heated at 60°C for 2.5h.

The mixture is allowed to cool to room temperature and excess of butanol is removed under vacuum (the temperature of evaporation must be under 60°C). 500mL of water are added. 90g of potassium carbonate are added very slowly (pH must be superior to 8). The layers are separated and the upon layer is sequentially extracted by 1*200mL of water and 200mL of a saturated solution of sodium chloride (350g/L). The upon layer is dried under 200g of anhydrous sodium sulphate for 1h and filtered on Büchner funnel to afford 842g (90%) of di n-butyl octanoate.

di n-Butyl sebacate

In a 2L-one neck flat-bottom flask equipped with a magnetic stirring bar, 424g (2.1mol) of sebacic acid and 777g (10.5mol, 5eq) of n-butanol are introduced. 124g (1.26mol, 0.6eq) of concentrated sulphuric acid are added and the mixture is heated at 60°C for 3h. First of all adipic acid is not soluble (white solid) but the mixture becomes limpid with the degree of conversion of the reaction.

The mixture is allowed to cool to room temperature and excess of butanol is removed under vacuum (the temperature of evaporation must be under 60°C). 500mL of water are added. 140g of potassium carbonate are added very slowly (pH must be superior to 8). The layers are separated and the upon layer is sequentially extracted by 1*200mL of water and 200mL of a saturated solution of sodium chloride (350g/L). The upon layer is dried under 180g of anhydrous sodium sulphate for 1h and filtered on Büchner funnel to afford 602g (91%) of di n-butyl sebacate.

di n-Butyl oleate

In a 2L-one neck flat-bottom flask equipped with a magnetic stirring bar, 727g (2.32mol) of oleic acid (90% pure) and 687g (9.28mol, 4eq) of n-butanol are introduced. 68g (0.696mol, 0.3eq) of concentrated sulphuric acid are added and the mixture is heated at 60°C for 2h.

The mixture is allowed to cool to room temperature and excess of butanol is removed under vacuum (the temperature of evaporation must be under 60°C). The two layers of the mixture are separated and 500mL of water are added to the upon layer. 100g of potassium carbonate are added very slowly (pH must be superior to 8). The layers are separated and the upon layer is sequentially extracted by 1*200mL of water and 200mL of methanol. The lower layer is dried under 180g of anhydrous sodium sulphate for 1h and filtered on Büchner funnel to afford 745g (95%) of di n-butyl oleate.

Simulants analysis

¹H NMR

Analysis are performed by Raffaele Lamanna.

- Frequency = 600MHz
- Solvent : Deuterated chloroform

IR

Simulants are analysed by FT/IR (ATR) by depositing a neat drop on the diamond.

- Number of scan : 8
- Scan interval : 650 to 4000 cm⁻¹
- Precision : 4 cm⁻¹

GC/FID

Simulants are dissolved in acetone (about 0.1%) and injected without further treatment.

Autosampler injector

- Temperature : 300°C
- Injected volume : 1 µl
- Split : 30

Column

- 100% DMS 15m*0.25mm*0.25 µm

Chromatograph :

- Oven : 1 min at 80°C, then 10°C/min until 325°C maintained for 15 min
- Flow (He) : 1 mL/min

Detector:

- Temperature : 300°C
- Attenuation : -6

GC/MS

Simulants are dissolved in acetone (about 0.1%) and injected without further treatment.

Autosampler injector

- Temperature : 280°C
- Injected volume : 1 µl
- Split : 20

Column

- RTX-5 Sil MS 30m*0.25mm*0.5 µm

Chromatograph :

- Oven : 1 min at 80°C, then 5°C/min until 300°C maintained for 10 min
- Flow (He) : 1 mL/min

Detector:

- Source temperature : 220 °C
- Transfer line temperature : 270 °C
- Solvent delay : 1 min
- Full scan : 45 et 500 m/z (1 scan/s)

Migration

A square piece of 1 dm² is set in a glass tube filled with 100mL of simulant. The film is cut in three parts to fit into the tube. Meshes are placed between films to ensure a contact of 1 dm² between the film and the liquid. The tube is put in an oven at 40°C for 10days. The sample is prepared in triplicate.

Migration assessment

A.4. Sample preparation

After migration, an exact amount of simulant (about 100mg) is weighted in a tube and 5mL of a mixture of tetrahydrofuran and water (85:15) is added with a precision pipette. The tube is closed and well mixed.

A.5. HPLC analysis

Autosampler injector

- Injected volume : 10 µl

Column

- C8 250mm*4.0mm*5 µm

Chromatograph :

- Flow : 1.0 mL/min
- Eluting gradient :

Time	% ACN	% H2O	% THF
0	50	30	20
10	50	30	20
15	80	0	20
25	80	0	20
30	50	30	20

40	50	30	20
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Detector:

- Source temperature : 220 °C
- Transfer line temperature : 270 °C
- Solvent delay : 1 min
- Full scan : 45 et 500 m/z (1 scan/s)

A.6. Chromatographic results

Simulant	Marqueur	Essai	m prise d'essai PE2 (mg)	Inj 1 PE2	Inj 2 PE2	Inj 3 PE2	m prise d'essai PE3 (mg)	Inj 1 PE3	Inj 2 PE3	
HO	C81 289nm	Blanc					102.4	0		
		1	99.1	104385	104718	104059	99.6	9516	8854	
		2	106.3	105800	102919	108385	102	11419	12536	
	3	102.9	107186	101579	103869	102.2	12143	11821		
	T326 349nm	Blanc						102.4	0	
		1						99.6	14096	14631
2							102	16959	15136	
3						102.2	16014	16524		
HOA	C81 289nm	Blanc					103.7	0		
		1	101.5	110386	107562	106071	103.6	12114	13381	
		2	102.3	109550	107836	111334	103.5	10935	11117	
	3	100.2	109150	105025	108345	100.9	12171	12664		
	T326 349nm	Blanc						103.7	0	
		1						103.6	16206	14515
2							103.5	16606	16178	
3						100.9	14859	16005		
HOB	C81 289nm	Blanc					-	-		
		1	105.3	107816	109334	106488	98.3	9224	10677	
		2	103.4	103636	102694	104643	102.1	9219	11693	
	3	99.8	99576	95424	101514	103.5	11326	12324		
	T326 349nm	Blanc						-	-	
		1						98.3	13579	15520
2							102.1	14782	16933	
3						103.5	15335	14875		
CL012	C81 289nm	Blanc					100.4	0		
		1	101	101374	104636	106926	101.5	12134	12687	
		2	101.6	105124	102211	101902	103.7	12452	12061	
	3	101.2	101284	103857	102548	103	10299	11254		
	T326 349nm	Blanc						100.4	0	
		1						101.5	16256	15262
2							103.7	16651	15228	
3						103	15320	14633		
CL013	C81 289nm	Blanc					102.6	0		
		1	102.2	107014	107526	104641	99.9	10387	12129	
		2	102.7	102984	108858	112378	103.3	11865	13498	
	3	102.6	101595	110759	105820	100.8	10626	11782		
	T326 349nm	Blanc						102.6	0	
		1						99.9	17811	17254
2							103.3	16061	18419	
3						100.8	17132	17533		
CL014	C81 289nm	Blanc					103.7	0		
		1	101.8	100321	97043	105201	102	13735	10739	
		2	102.4	103895	111676	110259	101.1	12152	9533	
	3	99.1	100553	100620	100684	99.3	10895	9902		
	T326 349nm	Blanc						103.7	0	
		1						102	19336	16574
2							101.1	18098	19589	
3						99.3	16144	18867		
CL015	C81 289nm	Blanc					100.5	0		
		1	102.9	97960	94082	95531	100.1	12712	12733	
		2	101.8	98162	93633	96741	99.5	8482	11172	
	3	102.3	99316	102939	103326	100.4	10689	10732		
	T326 349nm	Blanc						100.5	0	
		1						100.1	12712	17837
2							99.5	8482	17300	
3						100.4	10689	15941		
CL016	C81 289nm	Blanc					99.1	0		
		1	101	103399	107161	101829	102.6	16397	11341	
		2	100.2	97648	96265	96247	101.6	15906	10324	
	3	102.5	105938	104281	108413	100.7	17011	11297		
	T326 349nm	Blanc						99.1	0	
		1						102.6	16158	16158
2							101.6	12940	12940	
3						100.7	16650	16650		

100mg de simulant dilués dans 5 mL H₂O/THF 15:85

Part II: Development of NMR technique for the measurement of migration of chemicals from active packaging

1. Migration to simulants by NMR

In the deliverable D2B.5.1 we have developed a protocol for the measurement of chemical substances migrating from packaging system to food. In the present work we analysed by NMR the specific and overall specific migration in packaging systems additioned with nisin.

For the study of specific migration of nisin we need to individuate some NMR spectral lines which permit the identification and the quantification of the nisin even in a complex mixture. In figure 1 we show the ^1H NMR spectrum of a 2 mM solution of nisin in a mixture of H_2O and D_2O (9:1). Due to its complex chemical structure, the signals of nisin are spread all over the spectrum but some well identifiable signals are in the region between 6.5 and 9 ppm. These signals are attributed to NH protons of bound aminoacids in the peptidic chain [Lian1992]. To the solution we added, as internal standard, the 3-Trimethylsilylpropane sulfonic acid (DSS) at 1mM concentration. By integration of the NH resonance of nisin and the CH_2 resonance of DSS we estimate from the NMR spectrum a concentration of nisin of 2.1 mM which is in good agreement with the expected value.

We analysed 10 samples (see Table 1) prepared at LNE by migration experiments on the commercial INVOS film based on nisin.

Ref	Packaging	Simulant	Migration time	Temperature (°C)	Contact area (dm ²)	Solvent quantity (mL)	Volume/Surface (CM)
TFMI-OS005-1	INVOS	DBS	10	40	2	200	1
TFMI-SP023-B	Blank	water	10	40	0	200	-
TFMI-SP024-1	INVOS	water	10	40	1	200	2
TFMI-SP027-B	Blank	water	14	5	0	200	-
TFMI-SP028-1	INVOS	water	14	5	1	200	2
TFMI-SP026-1	INVOS	Ethanol 95%	10	40	1	200	2
TFMI-SP010-B	Blank	iso-octane	2	20	0	200	-
TFMI-SP011-1	INVOS	iso-octane	2	20	1	200	2
TFMI-SP014-B	Blank	iso-octane	18h	5	0	200	-
TFMI-SP015-1	INVOS	iso-octane	18h	5	1	200	2

Table 1: Migration test on nisin based films.

In figures 2 and 3, the proton NMR spectra of water samples taken in contact with INVOS polymer films for different times and temperatures are shown. From the spectra of figure 2 the overall specific migration is seen. Actually, in the high field region of the spectrum, between 0 and 3 ppm, we observe the presence of aliphatic chains, which can be tentatively assigned to fatty acids molecules, to polyethylene (PE) and lacquer's poly-vinylidene chloride (PVDC) monomers. The identification of these substances require the analysis of more samples by both 1D and 2D NMR experiments. In more details, in the sample taken at 40°C for 10 days the presence of triacylglycerols is evident. On the other hand, in the sample taken at 5°C for 14 days a different spectral pattern is observed in the same spectral region. The aspect of the spectra from blank samples is quite similar except for the total concentration and for the signal at 7 ppm which probably belongs to PVDC monomers. From figure 2 it appears a quite large variability of the amount and the quality of the contaminants extracted by water during the migration test at different temperatures. Its is quite difficult on the base of only two samples individuate the source of this variability. More experiments will be performed in the future in order to individuate the source of the above mentioned variability.

Figure 3 shows the same spectra but with a strong vertical expansion. From this figure it is possible to estimate the specific migration of nisin. In fact, the typical nisin resonances (especially those of aminoacids NH groups in the region 6.5 to 9 ppm) are seen in both INVOS

samples but not in the blanks as actually expected. The nisin signals in both INVOS sample are sufficiently intense to permit a preliminary quantification. By comparing the signal of nisin with that of DSS, as we did in figure 1 for the nisin standard, we estimate a concentration of nisin of 3.5 mM and 0.2 mM respectively for the samples taken at 40°C for 10 days and for the sample taken at 5 °C for 14 days.

In figure 4 the NMR spectrum of the ethanol extract is shown. The migration experiment was performed by taking the simulants in contact with the polymer for 10 days at 40°C (see Table 1). The NMR sample was prepared, according to the protocol described in the D2B5.1, by removing the simulant by evaporation under reduced pressure and then by dissolution into deuterated methanol (methanol d4). DSS 1 mM was added as reference. From the figure it is possible to estimate both specific and overall-specific migration. Except for the solvent residual signals no significant amount of contaminants can be individuated. On the other hand, in the region from 6.5 to 9.0 ppm, the signals of nisin migrated from the polymer are well visible.

Figure 5 shows the spectra of samples from the migration tests in iso-octane. According to the protocol previously established the NMR samples were prepared by evaporation of iso-octane under reduced pressure and successive dissolution in a smaller amount of deuterated chloroform. By this procedure we accomplish a concentration of the sample up to a factor of 15. From the spectra in figure 5 it appears the presence of aliphatic contaminants. However, these contaminants are present either in the INVOS and blank samples. By vertically expanding the spectra (see figure 6), we observe the presence of some low concentration compounds only in the INVOS samples. However, since their concentration is very low it is impossible to try an identification. We are planning to repeat the migration experiments in such a way to have more sample and increase the concentration factor.

From the analysis of the different simulants to be used in alternative to olive oil we have individuated Dibutyl sebacate as a good substituent.

In figure 7 we report the spectrum of the sample produced in a migration test in Dibutyl sebacate (DBS) of INVOS film. Accordingly to the previously described protocol the simulant was removed from the sample by Thin Layer Chromatography (TLC) using as mobile phase a mixture of CHCl₃ and CH₃OH (1:1).

The spot relative to DBS was removed from the plate and the remaining stationary phase eluted by the same solvent used as mobile phase in the chromatography. The protonated solvent was evaporated under reduced pressure and the sample dissolved in deuterated chloroform. From the spectrum we observe quite intense resonances in the aliphatic region probably due to film monomers and some low intensity signals which can be attributed to residual antioxidant and

plasticizers such as Irganox and Erucamide. As expected there is no presence of nisin in this extract.

2 Migration to food

The use of simulants, instead of real food, in migration experiments, if on one hand simplify the analytical process for the identification of migrants on the other cannot permit to have information on the eventual degradation of the migrants in contact with the real food. This issue is particular important in the migration of antimicrobial substances whose activity is concentration dependent. For this reason, we will try to identify, by NMR, the nisin directly in cheese matrices to be able to make specific migration experiments directly on the food.

In this section we described some preliminary results on the specific migration of nisin on hard cheeses. For the test we choose a hard Italian cheese called Provolone.

In figure 8 the solid state High Resolution Magic Angle Spinning (HR-MAS) spectrum of a piece of provolone is reported. The spectrum shows essentially the pattern of triacylglycerols with an high percentage of saturated fatty acids. The strong vertical expansion, shown in the inset of the figure, displays the signal of NH proton of proteins and polypeptides present in the cheese. In order to detect the other components of the provolone cheese we have made an extraction by a mixture of chloroform and water. After centrifugation at 10000 RPM for 10 min, three phases are separated: aqueous, organic and solid phases. Figure 9 shows the spectrum of the aqueous extract of provolone cheese. The most intense signals are due to lactate while the major part of the others belong to aminoacid groups. The vertical expansion of the spectrum (see the inset) clearly shows the presence of the typical resonance pattern of nisin. Figure 9 therefore demonstrate the possibility to detect the nisin directly in the aqueous cheese extract. To confirm the presence of nisin we performed a TLC with mobile phase constituted by a mixture of water and ethanol (1:1). The TLC plate of two samples of provolone and a standard of nisin is reported in the inset of figure 10. From this figure the chromatographic spot of nisin are well identified. The spectrum of the nisin TLC spot is shown in figure 10 together with the spectrum of the remaining part of the TLC plate. Experiments in which the provolone cheese is put in contact with the INVOS film are in progress.

Conclusions

Specific and overall specific migration were studied by NMR in nisin based films with four liquid simulants. Nisin was identified and quantified by NMR in both polar simulants (water and

ethanol). In addition, a NMR profile of some migrating contaminants was obtained in both polar and apolar simulants. Nisin resonances were identified in the spectrum of aqueous extract of provolone cheese and will be used to study the specific migration of nisin directly in the cheese.

Reference

[Lian1992]: L.Y. Lian, W.C. Chang, S.D. Morley, G.C.K. Roberts, B.W. Bycroft and D. Jackson. *Biochem. J.* (1992), **283**, 413-420

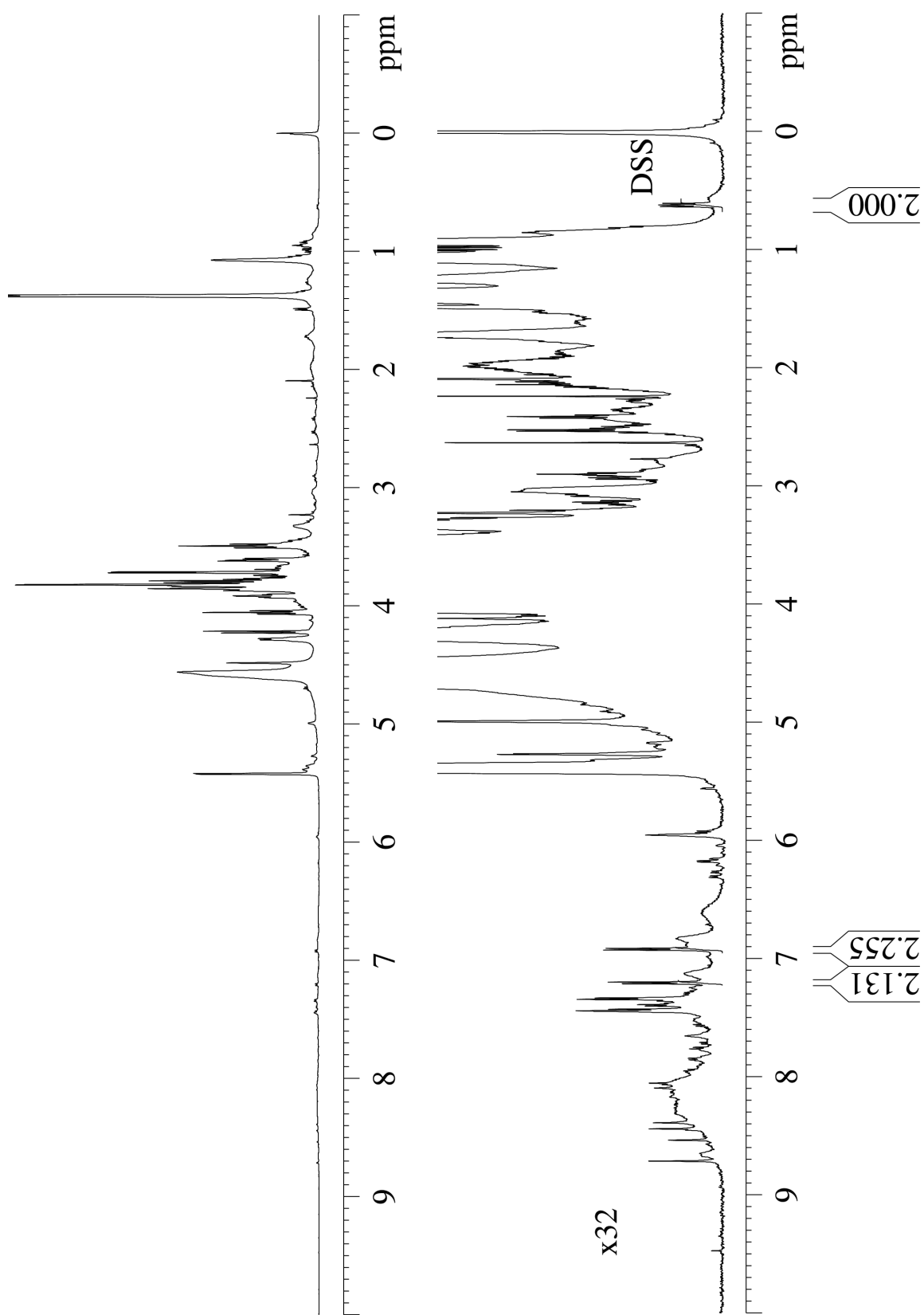


Figure 1: ^1H NMR spectrum of nisin standard in H_2O - D_2O (9:1).

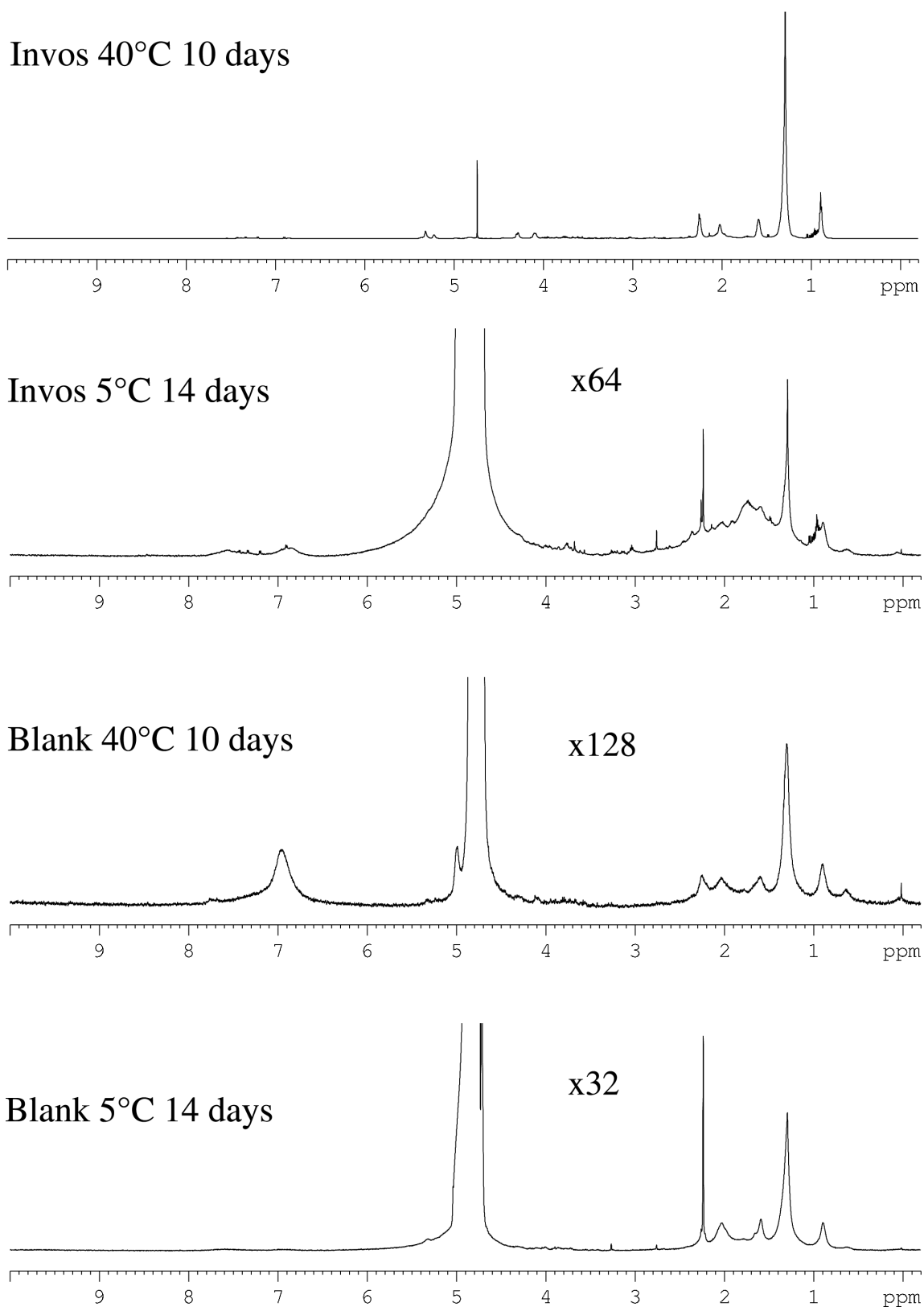


Figure 2: ^1H NMR spectrum of water extract, at different times and temperatures, of polymer films added with nisin.

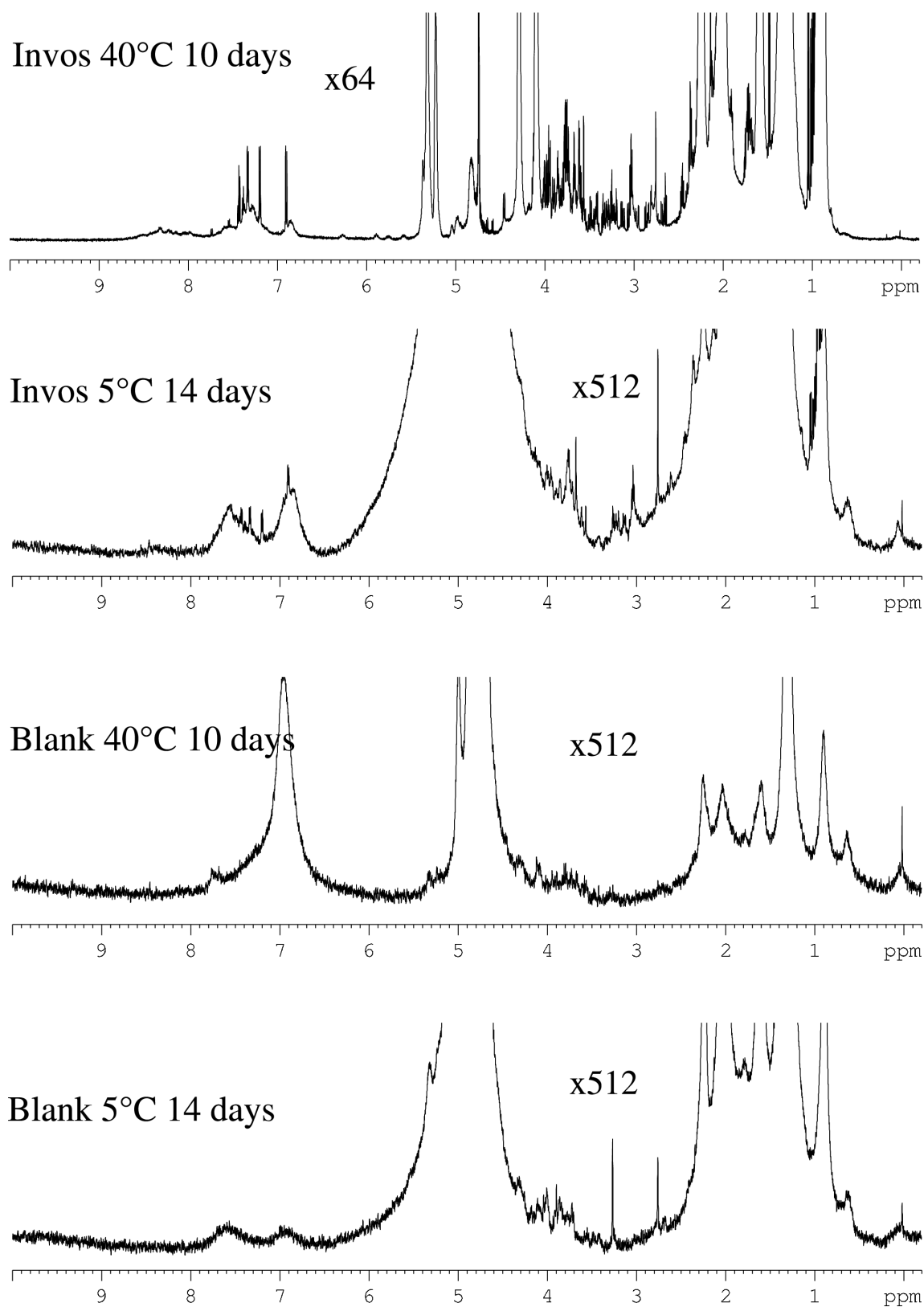


Figure 3: Vertical expansion of ^1H NMR spectrum of water extract, at different times and temperatures, of polymer films added with nisin.

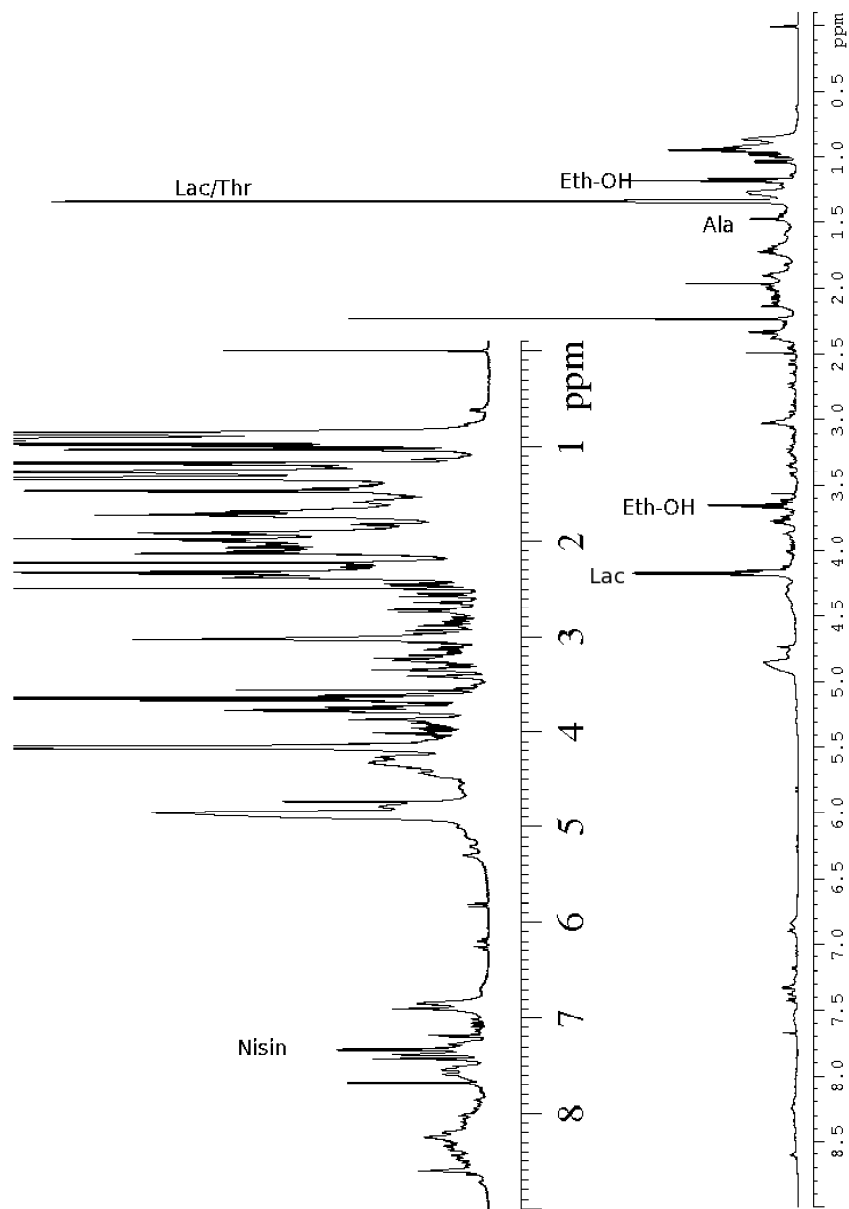


Figure 4: ^1H NMR spectrum of ethanol extract at 40 °C for 10 days of polymer films additioned with nisin.

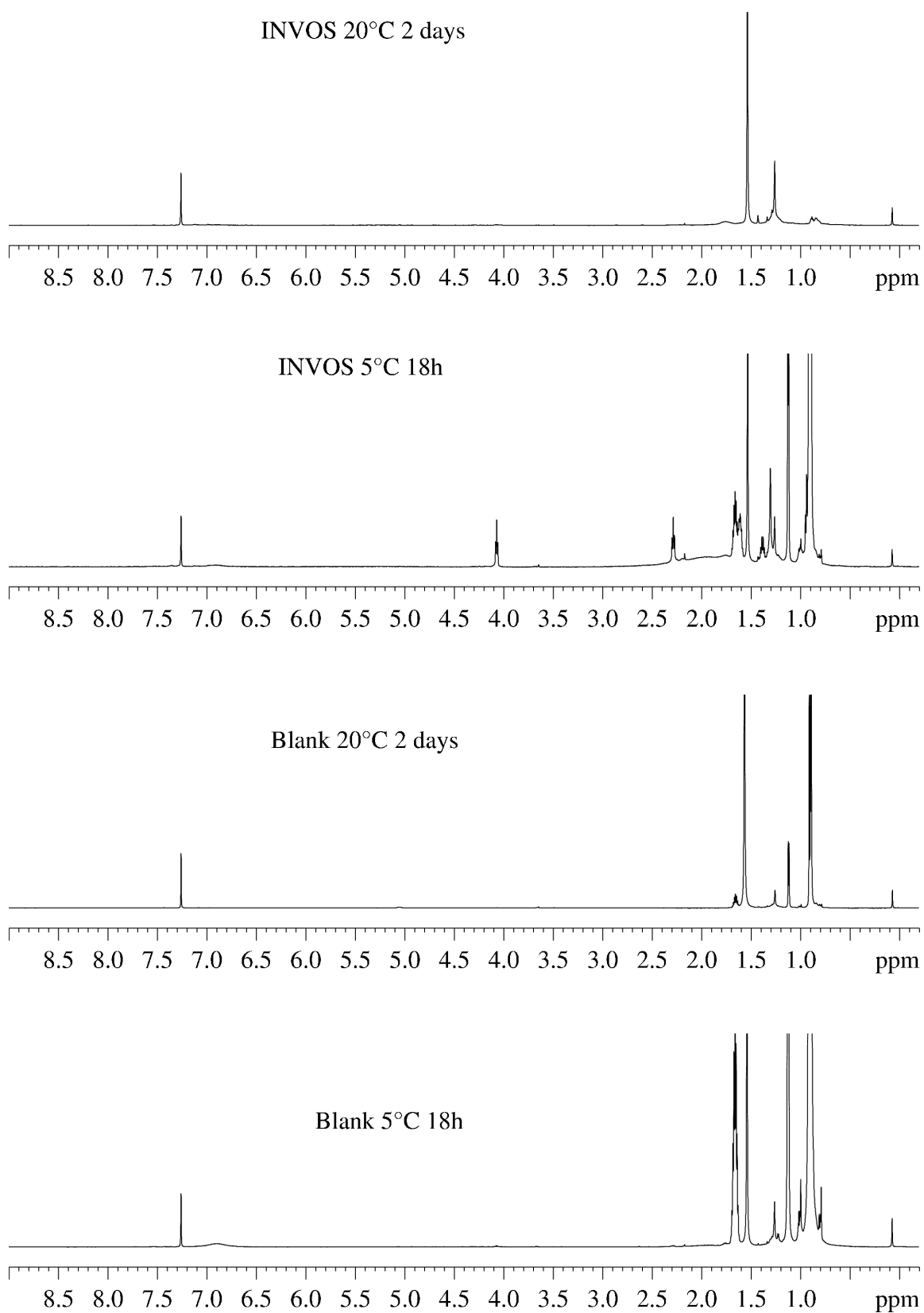


Figure 5: ^1H NMR spectrum of iso-octane extract, at different times and temperatures, of polymer films additioned with nisin.

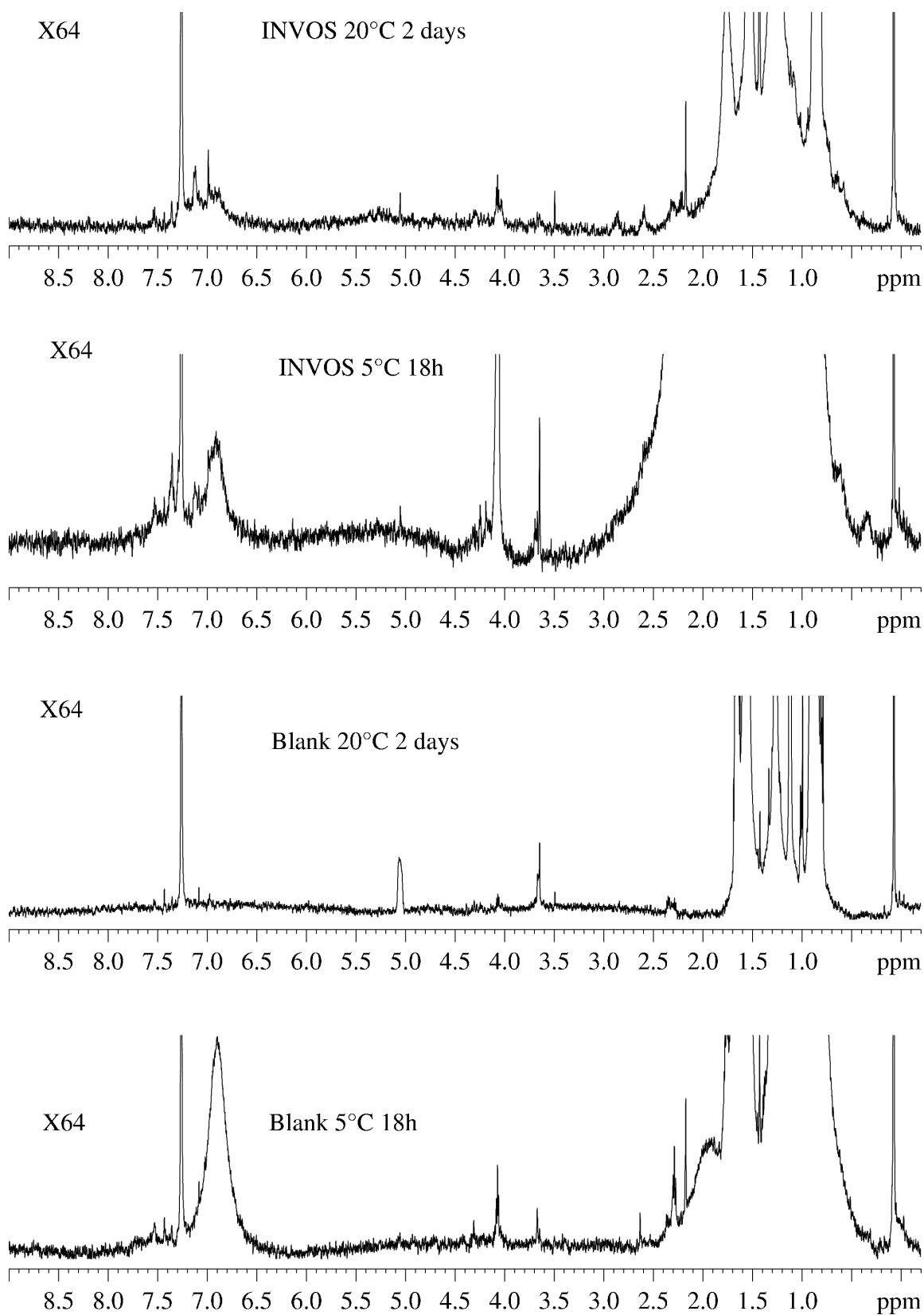


Figure 6: Vertical expansion of ¹H NMR spectrum of iso-octane extract, at different times and temperatures, of polymer films added with nisin.

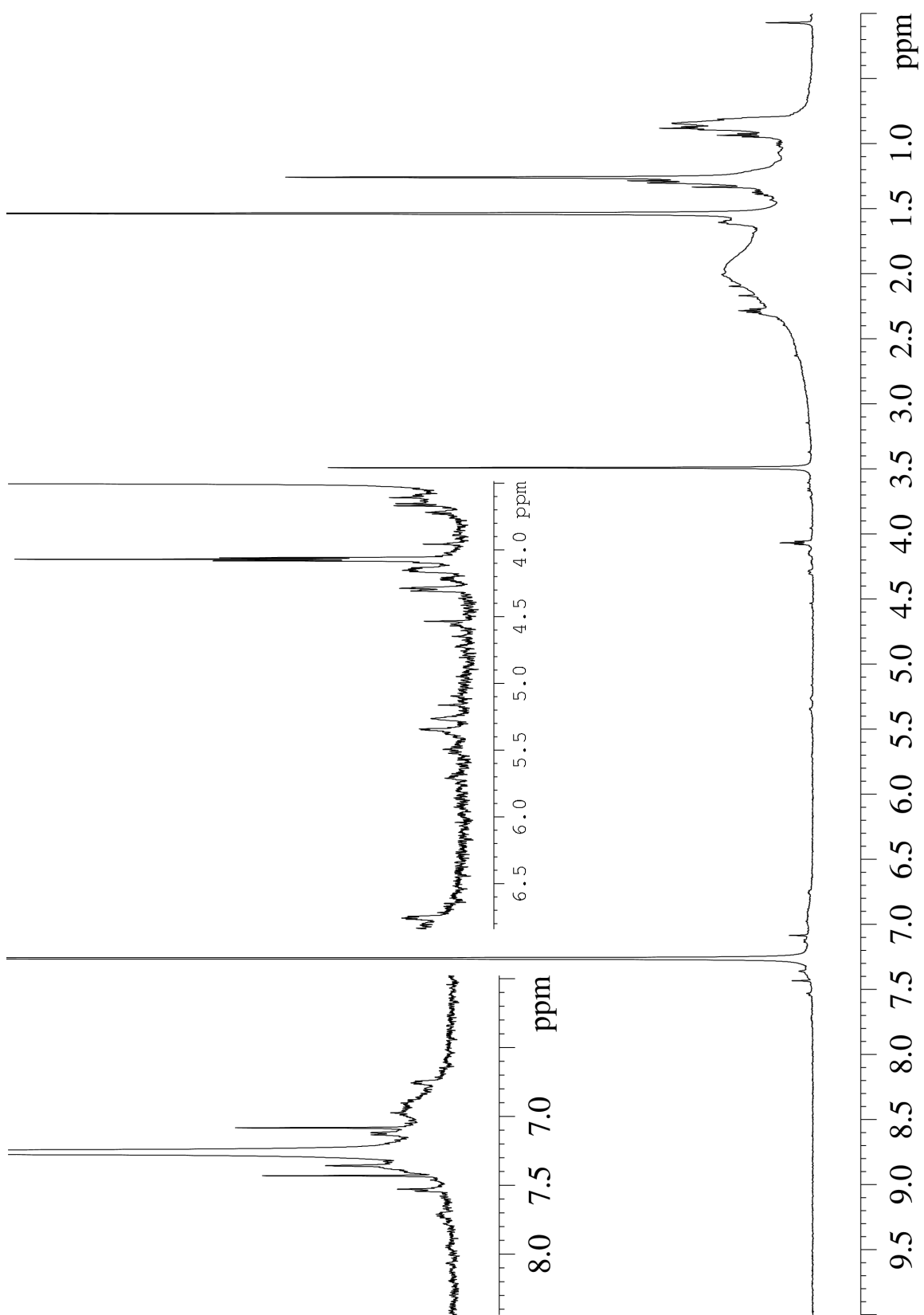


Figure 7: ^1H NMR spectrum of TLC fraction of DBS extract of polymer films added with nisin.

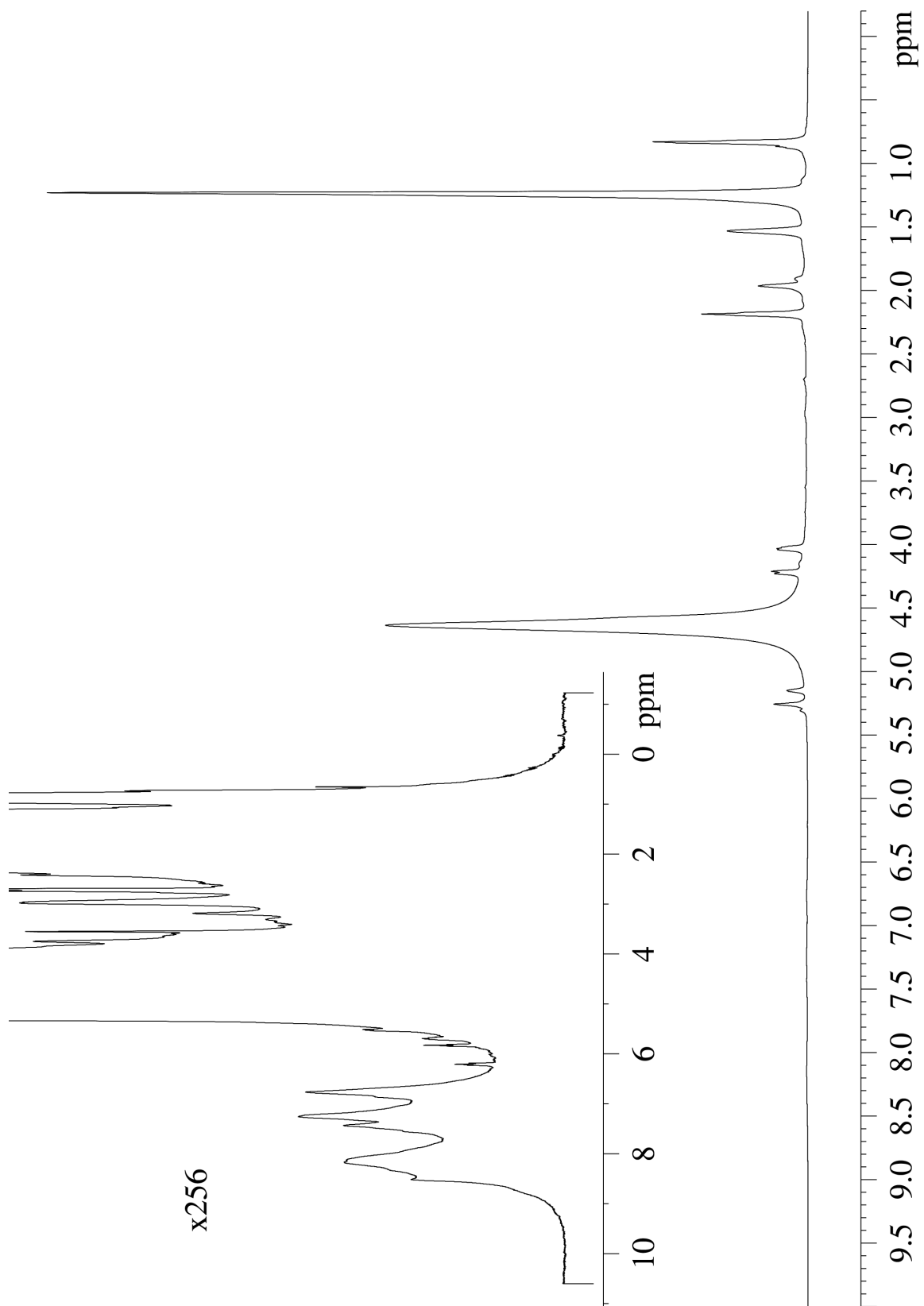


Figure 8: ^1H HR-MAS NMR spectrum of provolone cheese.

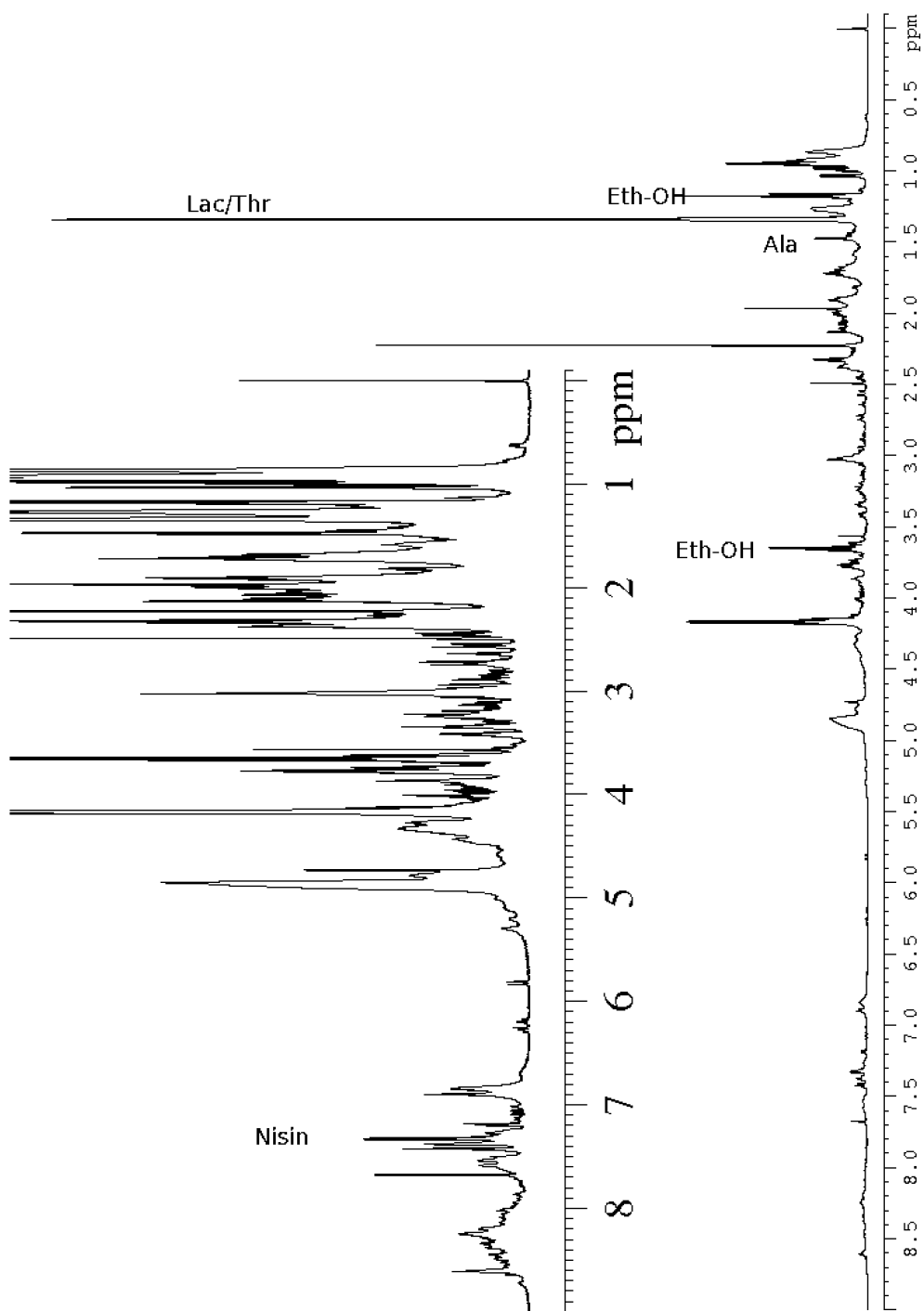


Figure 9: ^1H NMR spectrum of aqueous provolone cheese extract.

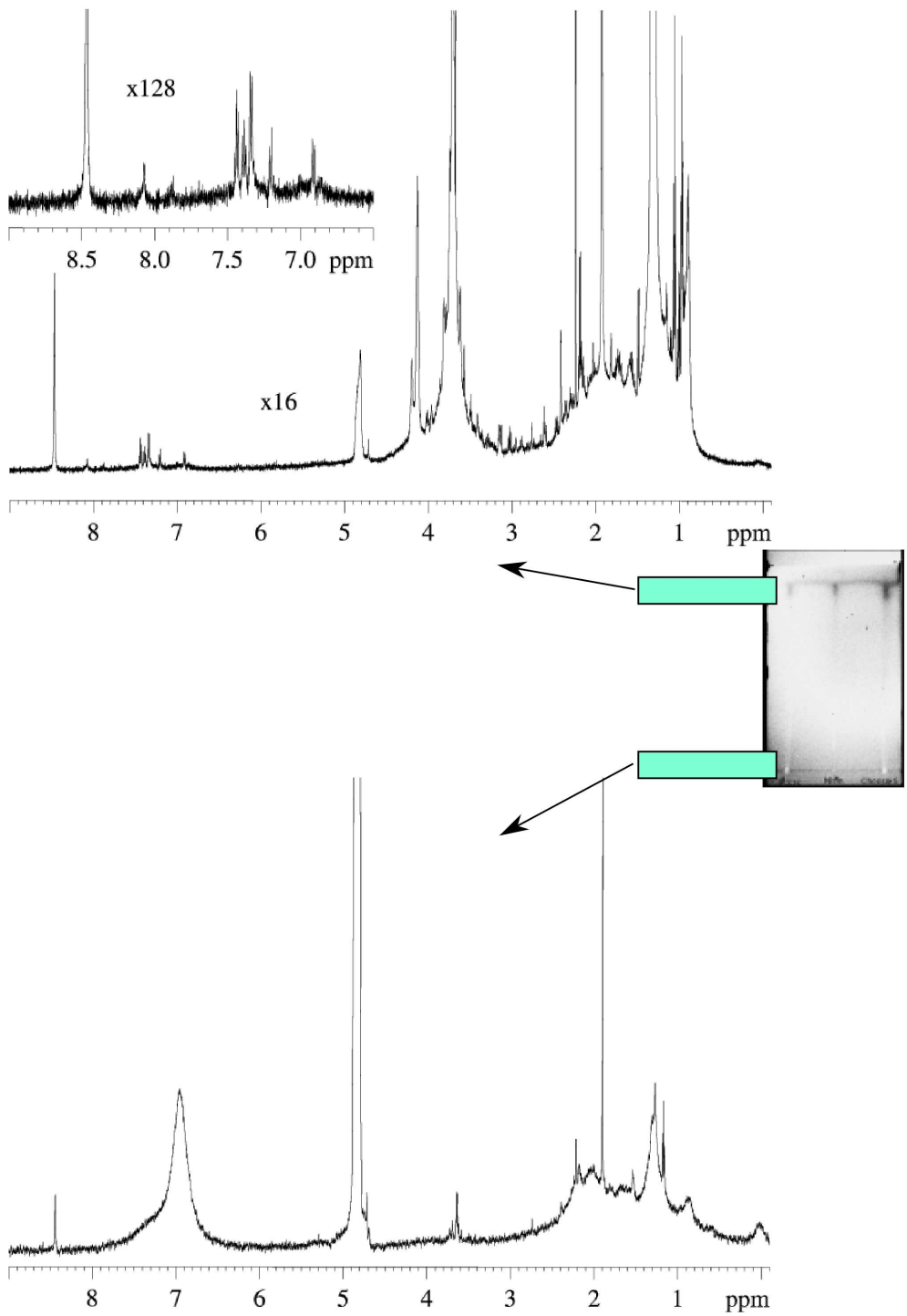


Figure 10: ^1H NMR spectrum of TLC fractions of aqueous extract of provolone cheese.