Field trial:
effect of the addition of stearic and palmitic acid to beeswax on the development of the worker bee brood

Final report: July 17 2018

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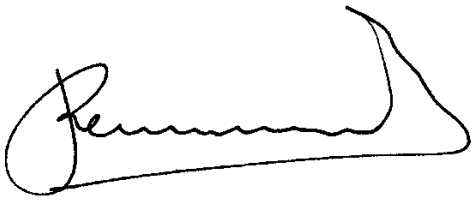

Introduction

In 2016, beekeepers alerted problems related to the poor development and dying-off of the bee brood after the insertion of new wax foundations which had been produced on an industrial scale. Following analysis, it appeared that the abnormal beeswax contained much higher levels of stearic and palmitic acid, which pointed to the addition of stearin. A 2017 field trial showed that adding 15 % or more stearin is responsible for the partial dying-off of the bee brood. (Reybroeck, 2017) ([1]). Data on the effects of adding lower concentrations to the beeswax are lacking. As such, the Federal Public Service for Public Health, Food Chain Safety and Environment asked the Flanders Research Institute for Agriculture, Fisheries and Food (ILVO) in Melle (BE) to carry out a new study in order to examine the effects of lower concentrations of two different stearins.

Objectives

To examine whether the addition of lower concentrations (2,5 to 10 %) on stearic and palmitic acid to beeswax leads to mortality of part of the worker bee brood ('shot brood'). Testing stearin of vegetal and animal origin in order to examine whether there is a difference in mortality in worker bee brood between a high concentration of stearic acid and a high concentration of palmitic acid. Generating results that can contribute to setting standards for establishing falsifications with saturated fatty acids.

Material and methods

Reference beeswax

Beeswax from Cameroon was used as a reference: 'Selected cast beeswax', Dadant Blatt, 41x26.3 cm, Lot 460, Best before 13/02/2020 (Bijenhof, Bissegem, BE) = ('Ref'). The wax contains very low levels of pesticides (analysis result is known) and complies with the melting point, acid and ester values as specified for pure beeswax ([2]).

Stearins

The stearins used (Radiacid, Oleon nv) are mixture of the (predominant) saturated fatty acids palmitic acid C16 and stearic acid C18, with a composition as shown in *Table 1*. Testing stearin of different origins enhances the certainty that no other (unknown) contaminations (e.g. in the palm oil) are causing the dying-off of bee brood.

*Table 1a. Composition of the stearins used in the field trials (**technical files**).*

Type stearin	CAS nr	Palmitic acid C16	Stearic acid C18	Others (<C16 and >C18)	Acid value (mg KOH/g)	Melting point
Radiacid 0407 (animal origin)	67701-03-5	25-35 %	60-71 %	≤8 %	200-210	57-61°C

([1]) Reybroeck, Wim 2017. Field trial: effect of the addition of a mixture of stearic and palmitic acid (called stearin) to beeswax on the development of the worker bee brood. Final report: June 30, 2017. ILVO, Melle, 1-14.

([2]) As established in Regulation (EU) No. 231/2012 laying down specifications for food additives listed in Annexes II and III to Regulation (EC) No. 1333/2008 of the European Parliament and of the Council (for acid value and saponification value), and in accordance with the relevant literature.

Type stearin	CAS nr	Palmitic acid C16	Stearic acid C18	Others (<C16 and >C18)	Acid value (mg KOH/g)	Melting point
Radiacid 0464 (origin palm stearin)	67701-03-5	54-68 %	31-43 % (C18+C18:1)	≤6 %	209-214	54-56°C
Radiacid 0417 (origin palm oil)	67701-03-5	42-49 %	50-55 %	≤4 %	205-211	Ca. 56 °C

*Table 1b. Composition of the stearins used in the field trials (**producer's analysis**).*

Type stearin	CAS nr	Palmitic acid C16	Stearic acid C18	Others (<C16 and >C18)	Acid value (mg KOH/g)	Melting point
Radiacid 0407 (animal origin)	67701-03-5	29,9 %	63,2 %	6,9 % mainly (>1 %) C14, C17, C20	205,4	(tech. file: 57-61°C)
Radiacid 0464 (origin palm stearin)	67701-03-5	60,0 %	37,6 %	2,4 % mainly (>1 %) C14	211,4	54,9 °C
Radiacid 0417 (origin palm oil)	67701-03-5	43,5 %	54,2 %	2,3 % mainly (>1 %) C14	206,1	(tech. file: Ca. 56 °C)

Cast wax foundations with added stearin
The reference beeswax was re-melted in a Pyrex beaker from which newly cast wax foundations ('T') where made in the laboratory in a wax foundation mould with water cooling 'Normal 350 x 200' (Graze, Weinstadt, DE). The cast wax foundations (‘T’) have the same composition as the purchased wax foundations.

Saturated fatty acids C16-C18 were added to the wax in increasing weight concentrations, namely 2,5; 5; 7,5 and 10 %. Each time, wax foundations were cast from the various wax compositions, in the same way as (‘T’).
The cast wax foundations obtained were: saturated fatty acids C16-C18 (Radiacid 0407; high in stearic acid content): 2,5 % (‘K’), 5 % (‘L’), 7,5 % (‘M’) and 10 % (‘N’).
The cast wax foundations obtained were: saturated fatty acids C16-C18 (Radiacid 0464; high in palmitic acid content): 2,5 % (‘O’), 5 % (‘P’), 7,5 % (‘Q’) and 10 % (‘R’).
Additionally a wax foundation was cast, containing 15% Radiacid 0417 (‘S’) which was also used in the 2017 field trial ([1]).

Al of the samples of the beeswax mixed with saturated fatty acids C16-C18 were analysed by the laboratory Ceralyse (Celle, DE), which is specialised in analysing beeswax, for acid and ester value and saponification number. The reference beeswax (‘T’) en wax (‘S’) with 15 % stearin were also analysed for the hydrocarbons content. A gas chromatographic fingerprint was also carried out on these samples by Ceralyse (see below 'Results and discussion').

Apiary and bee colonies
The apiary is situated at Brusselsesteenweg 370, Melle (BE). Four Dadant Blatt bee hives were used, populated with the European honey bee (*Apis mellifera*). Two queens (*Carnica* Troiseck) were bred by Jacques Levrau and fertilised in Kreverhille in 2016, one queen (F1 - *Carnica* Troiseck) was bred by André Decaluwe and fertilised in 2016 at his apiary while the last queen is a young queen (2017), a daughter of a queen of Willy Geirnaert and fertilised in Melle.

Experimental design
Purchased wax foundations made of good beeswax were used ('Ref'). These were cut to the size of Dadant Blatt super frames, with interior dimensions of 13x41 cm.

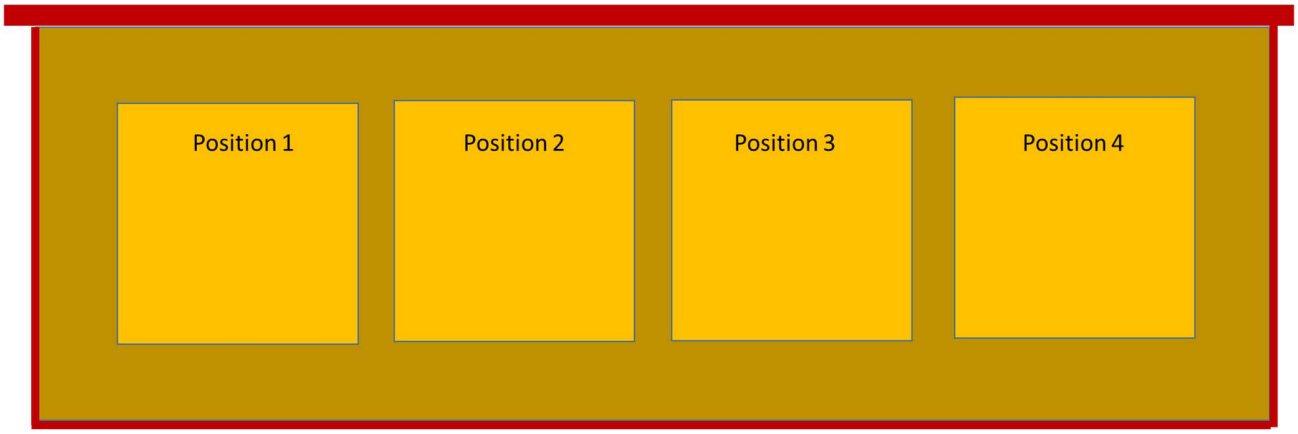

Fig. 1. Scale diagram of a Dadant Blatt super frame with 4 pieces of wax foundation to be tested (1 reference wax foundation and 3 different situations).

Four openings of 8x8 cm (= 64 cm^2) were applied to these wax foundations, into which a piece of the cast wax foundation to be tested could fit (Figure 1). The wax foundation with the openings and the pieces of cast wax foundation were held in place by a suitably positioned and fused small thread. For each test frame, a piece of cast reference wax was applied as a kind of internal check.

Each test frame was hung separately in a bee colony in the super, to enable the wax honeycomb to be built up by the worker bees. After 2 to 3 days, the queen was located in the bee colony in question, and enclosed on the test frame with built-up honeycomb, by placing a small frame with a flat queen excluder on both sides of the test frame. The queen confinement cage was placed in the middle of the super. That way, the queen can only lay eggs in the cells of the frame in question. After 2 to 3 days, the queen was removed from the frame and released into the brood area below, under a queen excluder, so that there could be no disruption by the subsequent relaying of eggs in cells by the queen. The egg-filled test frame was checked each time to see whether an egg had been laid in each cell. After these checks, the test frame was replaced in the colony for the further development of the worker bee brood, which was also monitored. At the moment that all the larvae had pupated and the brood was sealed, photo-recordings was carried out. In this phase, a distinction can clearly be made between open cells in which the egg/larva has died, and the sealed cell with a living pupa. To this end, the detailed photographs of each situation were printed and the open and closed cells were manually counted. As such, the survival percentage could be calculated. Given that a bee honeycomb is built up on both sides of the wax foundation (middle wall), results were obtained for each test frame for both the left and right side, in other words results for around 210 cells for each side.

This approach makes it possible to rule out as many external influence factors as possible (queen, food, etc.) on the survival of the brood. The position within the frame itself may have an influence; for this reason, the positions of the pieces of wax foundation were changed when the test was repeated: each situation was at least tested *in duplo*. At the same time, the parallel situation was tested in a second bee hive (with a different queen). In addition, the pieces of wax foundation were mounted in another position: position 1 to position 3; position 2 to position 4; position 3 to position 1; position 4 to position 2.

In Table 2, an overview is shown of the different test frames and the tested wax foundations.

Table 2. Overview of the different test frames, the position of the tested wax foundations and the date on which the queen was placed in confinement.

Test frame	Side	Wax foundation				Starting date
		Position 1			Position 1	
7	left	T	K	O	L	12/05/2018
	right	L	O	K	T	
8	left	T	P	M	Q	13/05/2018
	right	Q	M	P	T	
9	left	T	N	R	B	13/05/2018
	right	B	R	N	T	
10	left	O	L	T	K	12/05/2018
	right	K	T	L	O	
11	left	M	Q	T	P	13/05/2018
	right	P	T	Q	M	
12	left	R	S	T	N	13/05/2018
	right	N	T	S	R	
7'	left	T	K	O	L	22/06/2018
	right	L	O	K	T	
8'	left	T	P	M	Q	22/06/2018
	right	Q	M	P	T	
9'	left	T	N	R	B	22/06/2018
	right	B	R	N	T	
10'	left	O	L	T	K	24/06/2018
	right	K	T	L	O	
11'	left	M	Q	T	P	24/06/2018
	right	P	T	Q	M	
12'	left	R	S	T	N	24/06/2018
	right	N	T	S	R	

Legend:
Blue: results cancelled due to incomplete egg-filling;
Yellow: results cancelled due to the presence of nectar/half-ripe honey;
Green: results based on a count of a part of the inserted piece of beeswax.

Tests were carried out over the period May 12 – July 6, 2018. In May and the beginning of June there was significant nectar flow so that the queens often could not lay eggs in the freshly built frames as the harvesting bees deposited nectar and half-ripe honey in the cells. The counts of the positions in question were cancelled and were not included in the report (marked in yellow).

On two test frames, only part of the cells were counted (marked in green in table 2) as part of the cells were filled with honey instead of eggs at the moment the queen was liberated.
Strangely enough, in test frame 10', for position L, the cells were poorly filled with eggs. The results for this situation were cancelled (marked in blue).

Results and discussion

a) Results of analyses of wax samples of reference wax, and from wax mixed with stearin (Ceralyse, Celle, DE)

The results of the analysis at Ceralyse of acid value, ester value and saponification value in samples ('K') to ('T') and total hydrocarbon content in samples ('S') and ('T') are shown in *Table 3*.

Table 3. Ceralyse analysis results

	Acid value	Ester value	Saponification value (mg KOH/g)	Total hydrocarbons	C16 & C18 added
Normal values ([2])	17-24	70-80	87-104	13-13,5 % (Afrikaanse origine)	---
Sample ('T')	19,5	75,3	94,8	13,7 %	0% ('Ref') = ('T')
Sample ('K')	23,9	72,9	96,8		2,5 % R0407 (97,5 % 'Ref')
Sample ('L')	27,6	71,0	98,6		5 % R0407 (95 % 'Ref')
Sample ('M')	33,8	67,0	98,6		7,5 % R0407 (92,5 % 'Ref')
Sample ('N')	38,1	65,3	103,4		10 % R0407 (90 % 'Ref')
Sample ('O')	23,6	72,5	96,1		2,5 % R0464 (97,5 % 'Ref')
Sample ('P')	28,1	71,5	99,5		5 % R0464 (95 % 'Ref')
Sample ('Q)	34,0	66,5	104,5		7,5 % R0464 (92,5 % 'Ref')
Sample ('R')	39,4	65,7	105,1		10 % R0464 (90 % 'Ref')
Sample ('S')	47,3	65,0	112,3	11,5 %	15 % R0417 (85 % 'Ref')

From the results, it appears that the addition of stearin results in an increase in acid value and in the saponification number. On the other hand, both the ester value and the percentage of hydrocarbons decline. However this also shows that adding (especially low percentage) stearin not always causes a deviation to normal values for acid and ester value and saponification value([2], deviations marked in color). As an example, adding 10 % Radiacid 0407 of animal origin does not lead to exceeding of the norm set for saponification number.

In the results, Ceralyse observed that the hydrocarbons content in the reference wax is slightly

higher than normal for beeswax of Cameroonian origin ('T') but is in the range of the accuracy of the method (± 0,3 %). This can also be attributed to the addition of a very small quantity of hydrocarbons (such as paraffin) or to the fact that the beeswax largely consists of old honeycombs, meaning that this content can increase slightly. From literature, it appears that such small deviations do not have any impact on the development of the bee brood.

A gas chromatographic fingerprint was also carried out at Ceralyse. In the chromatograms, the peaks for palmitic and stearic acid are clearly present in the samples ('B'), ('D') and ('F'). The chromatograms of the reference wax ('A') and wax sample ('D') are shown in Annex 1.

A gas chromatographic fingerprint was also carried out at Ceralyse. In the chromatograms, the peaks for palmitic and stearic acid are clearly present in the samples ('K') to ('N') and ('O') to ('R'). In ('O') to ('R') palmitic acid peaks are more pronounced (palm origin), compared to the stearic acid. The chromatograms of the reference wax ('T') and wax samples ('K') to ('R') are shown in Annex 1.

b) Results of brood mortality

All the inserted wax foundations were well built-up by the bees, and then laid with eggs by the queens. If not all cells were laid with eggs, this was recorded. Dying-off of the brood occurred at the start of the larval stage. The counts were made at the moment the surviving brood had pupated (sealed cells), which made the counting easier. The results of the counts are shown in Table 4. In addition, survival was also calculated with regards to the survivals obtained with the reference wax (Cameroon) on the same side as the test frame. No dying-off during the pupa stage was observed: the pupae developed into imago (adult bees).

Table 4. Results of the survival of the worker bee brood in beeswax of various composition.

Frame	Side	Situation	Survival (%)	Average survival (%)	Survival w.r.t. reference (%)	Average survival w.r.t. reference (%)	Code
7'	L	T	74,5	79,4	100	100	Ref Cameroon
	R		84,2		100		
	L	K	83,2	86,8	111,6	109,5	2,5 % Radiacid 0407
	R		90,4		107,3		
	L	O	83,2	85,1	111,16	107,2	2,5 % Radiacid 0464
	R		87,0		103,30		
	L	L	73,3	71,7	98,4	90,9	5 % Radiacid 0407
	R		70,1		83,3		

Frame	Side	Situation	Survival (%)	Average survival (%)	Survival w.r.t. reference (%)	Average survival w.r.t. reference (%)	Code
8'	L	T	71,6	73,2	100	100	Ref Cameroon
	R		74,7		100		
	L	P	83,2	81,0	116,2	110,9	5 % Radiacid 0464
	R		78,7		105,5		
	L	M	51,6	57,8	72,0	78,8	7,5 % Radiacid 0407
	R		63,9		85,6		
	L	Q	50,2	49,9	70,1	68,2	7,5 % Radiacid 0464
	R		49,5		66,3		
9 Spring	L	T	74,7	70,1	100	100	Ref Cameroon
	R		65,4		100		
	L	N	25,0	25,7	33,5	37,0	10 % Radiacid 0407
	R		26,4		40,4		
	L	R	24,6	25,0	32,9	35,9	10 % Radiacid 0464
	R		25,4		38,8		
	L	S	23,1	29,2	30,9	42,5	15 % Radiacid 0417
	R		35,3		54,0		
10 Spring	L	O	--	87,0	--	99,7	2,5 % Radiacid 0464
	R		87,0		99,7		
	L	L	86,0	88,9	101,7	103,5	5 % Radiacid 0407
	R		91,8		105,2		
	L	T	84,5	85,9	100	100	Ref Cameroon
	R		87,3		100		
	L	K	--	--	--	--	2,5 % Radiacid 0407
	R		--		--		
10'	L	O	75,1	67,7	100	86,6	2,5 % Radiacid 0464
	R		60,2		73,2		
	L	L	--	--	--	--	5 % Radiacid 0407
	R		--		--		
	L	T	75,1	78,7	100	100	Ref Cameroon
	R		82,2		100		
	L	K	59,4	61,0	79,1	77,6	2,5 % Radiacid 0407
	R		62,5		76,0		
11'	L	M	55,2	55,3	67,9	67,9	7,5 % Radiacid 0407
	R		55,3		67,9		
	L	Q	52,0	50,3	64,0	61,8	7,5 % Radiacid 0464
	R		48,6		59,6		
	L	T	81,25	81,4	100	100	Ref Cameroon
	R		81,45		100		
	L	P	52,5	48,2	64,6	59,2	5 % Radiacid 0464
	R		43,8		53,7		

Frame	Side	Situation	Survival (%)	Average survival (%)	Survival w.r.t. reference (%)	Average survival w.r.t. reference (%)	Code
12'	L	R	18,6	19,7	24,1	23,9	10 % Radiacid 0464
	R		20,8		23,6		
	L	S	26,2	33,1	34,1	39,7	15 % Radiacid 0417
	R		39,9		45,2		
	L	T	76,9	82,6	100	100	Ref Cameroon
	R		88,2		100		
	L	N	11,3	12,9	14,7	15,6	10 % Radiacid 0407
	R		14,4		16,4		

The results of the replications per situation are summarised in Table 5. Survival was also calculated with regards to the survivals obtained with the reference wax on the same side as the test frame. These results are shown in Table 6.

Table 5. Summary of the results of the survival of the worker bee brood in beeswax of various composition (per situation).

Survival (%) for each type of wax									
Ref.	Radiacid 0407				Radiacid 0464				Radiacid 0417
T	K 2,5%	L 5%	M 7,5%	N 10%	O 2,5%	P 5%	Q 7,5%	R 10%	S 15%
79,4	86,8	71,7	57,8	25,7	85,1	81,0	49,9	25,0	29,2
73,2	61,0	88,9	55,3	12,9	87,0	48,2	50,3	19,7	33,1
70,1					67,7				
85,9									
78,7									
81,4									
82,6									
Average survival (%) for each type of wax									
78,8	73,9	80,3	56,6	19,3	79,9	64,6	50,1	22,4	31,2

Table 6. Summary of the results of the survival of the worker bee brood in beeswax of various composition (per situation) related to the survival in reference wax.

Survival (%) with regards to the survivals obtained with the reference wax for each type of wax									
Ref.	Radiacid 0407				Radiacid 0464				Radiacid 0417
T	K 2,5%	L 5%	M 7,5%	N 10%	O 2,5%	P 5%	Q 7,5%	R 10%	S 15%
100,0	109,5	90,9	78,8	37,0	107,2	110,9	68,2	35,9	42,5
100,0	77,6	103,5	67,9	15,6	99,7	59,2	61,8	23,9	39,7
100,0					86,6				
100,0									
100,0									
100,0									
100,0									
Average survival (%) with regards to the survivals obtained with the reference wax for each type of wax									
100,0	93,6	97,2	73,4	26,3	97,8	85,1	65,0	29,9	41,1

Discussion

In the test frames with limited addition (2,5 % and 5 %) of C16&C18, mortality is not significantly higher. In some cases mortality is even lower than in the reference wax. In all other test frames (addition of 7,5; 10 or 15 % C16&C18) survival of the worker bee brood was significantly lower than in the reference wax.

Besides, in the reference wax, the worker bee brood does not show a 100 % survival either. Remarkably, the dying-off of the brood occurs in cells where the frame wire was melted in. On an average, 21,2 % of the eggs do not develop into pupas. In last year's field trials (Reybroeck, 2017) mortality was 18,5 %.

When comparing the mortality caused by the different types C16&C18, we see that adding 10 % Radiacid 0407 (73,7 %) and 0464 (70,1 %) causes a higher mortality than adding 15 % Radiacid 0417(68,8 %).

In 2017, mortality caused by 15 % Radiacid 0417 amounted to 65,5 % (Reybroeck, 2017) which shows that the test generates repeatable results.

Regarding standards for wax intended for use in apiculture, respecting an acid value between 17-24 and an ester value between 70-80 can certainly be advised for checking bee wax purity on falsification by the addition of saturated fatty acids. A standard for the saponification number makes less sense. The question remains whether this offers sufficient guarantees for preventing brood mortality. A GC-fingerprint establishing the absence of falsification offers more reliability.

Conclusion

The results show that beeswax with 7,5 % (or higher percentages) C16&C18 added, is certainly not suitable for use as a raw material for the production of wax foundations for use in apiculture. Based on the average results, there are already negative effects when adding 2,5 and 5 % C16&C18, albeit to a limited extent.

Remarks regarding the results

Attention is drawn to the fact that the test was carried out with very pure reference wax, and in healthy bee colonies. In many practical cases, beeswax still contains a certain percentage of added hydrocarbons (for example paraffin) and a higher content of pesticide residues which may have an additional (mutually reinforcing) negative influence on the development of the bee brood.

Other types of stearin (for example of a different origin and with a different ratio of palmitic acid/stearic acid) can generate a different result.

The study only provides a snapshot of the effect of stearin over a very short timeframe of the life cycle of a bee hive. With significant brood losses, the chances of survival of a bee colony are seriously curtailed.

Annex 1 . Chromatograms

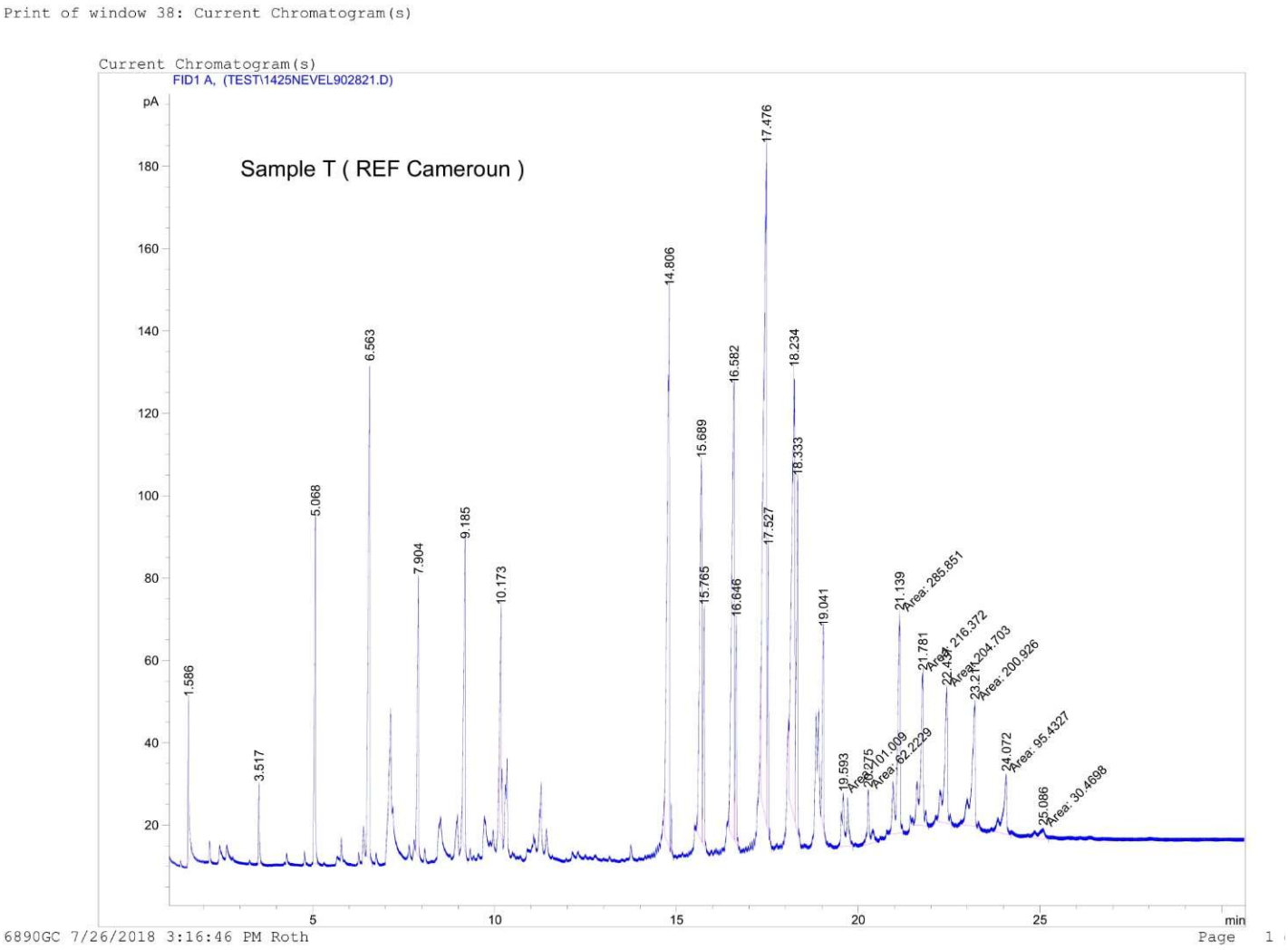

Fig. 2. Chromatogram of the reference wax ('T').

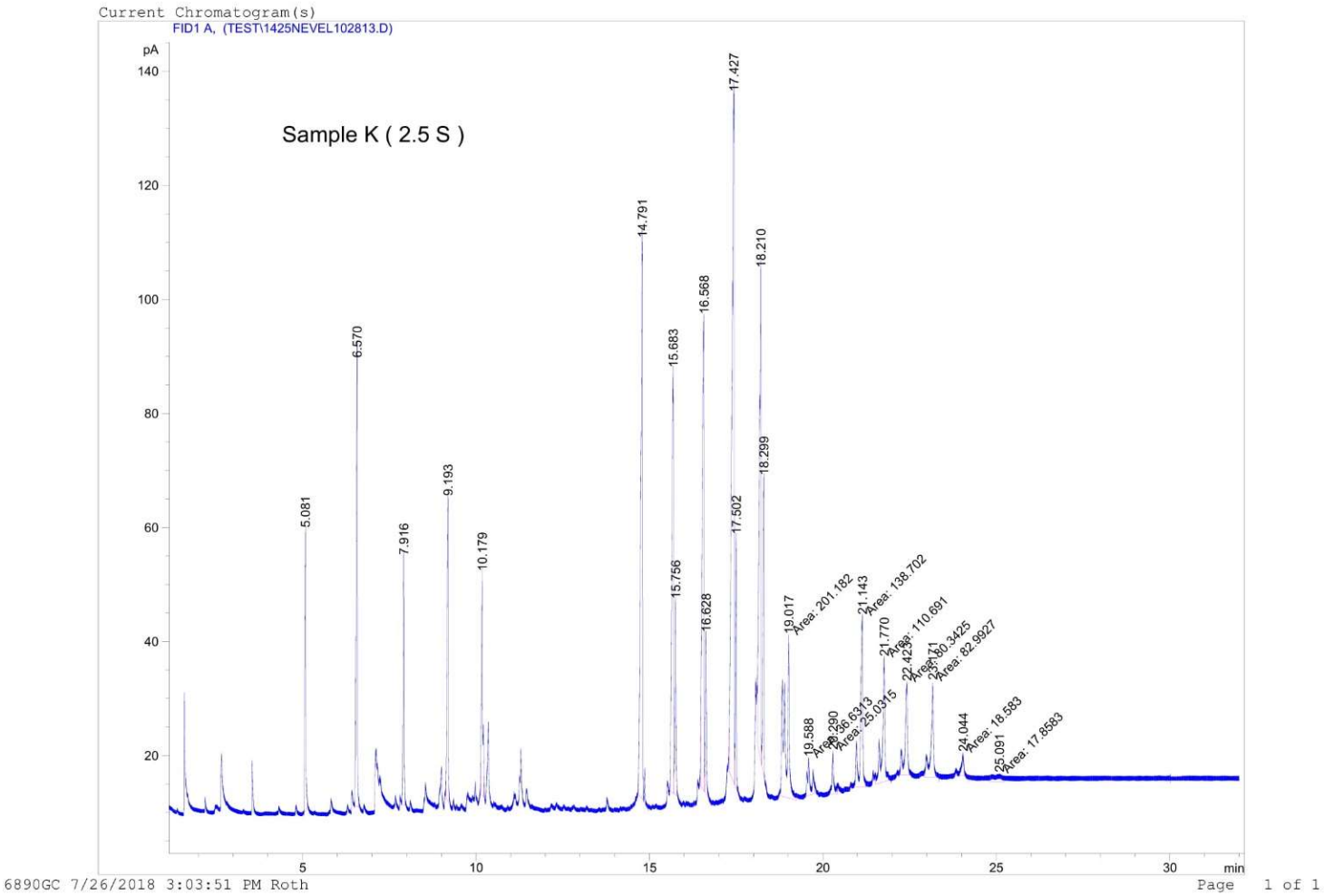

Fig. 3. Chromatogram of the wax ('K') with 2,5 % Radiacid 0407 (animal origin).

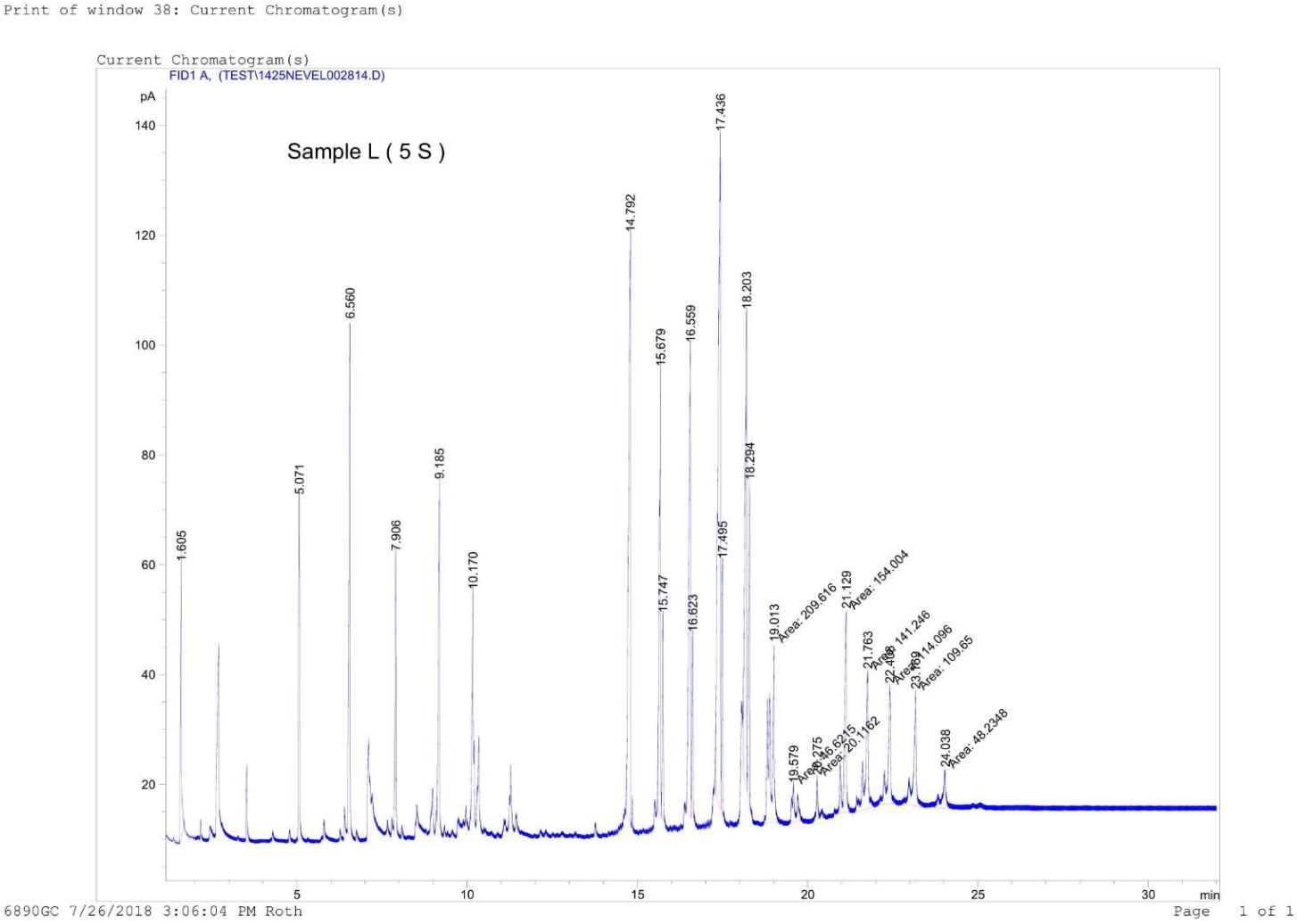

Fig. 4. Chromatogram of the wax ('L') with 5 % Radiacid 0407 (animal origin).

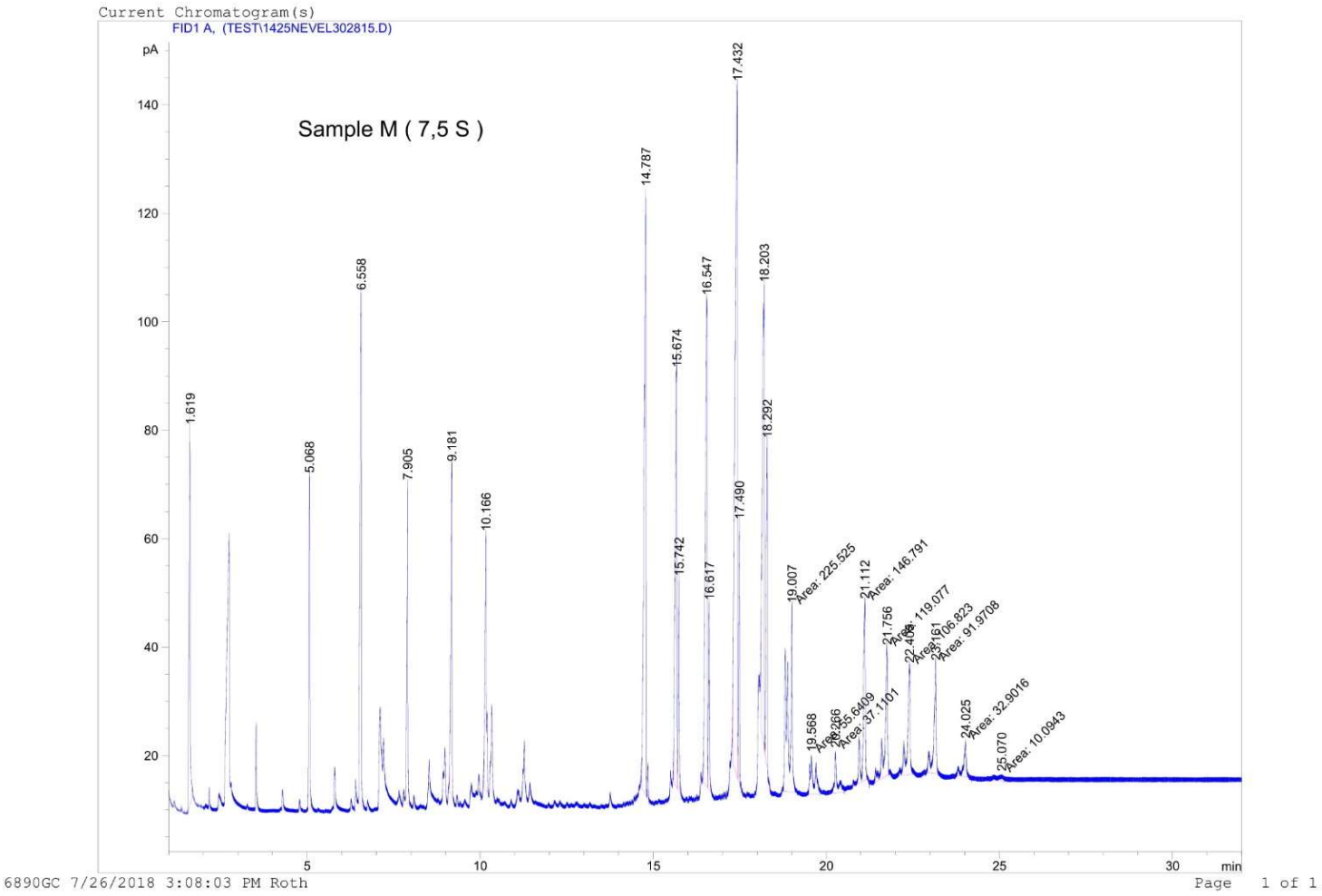

Fig. 5. Chromatogram of the wax ('M') with 7,5 % Radiacid 0407 (animal origin).

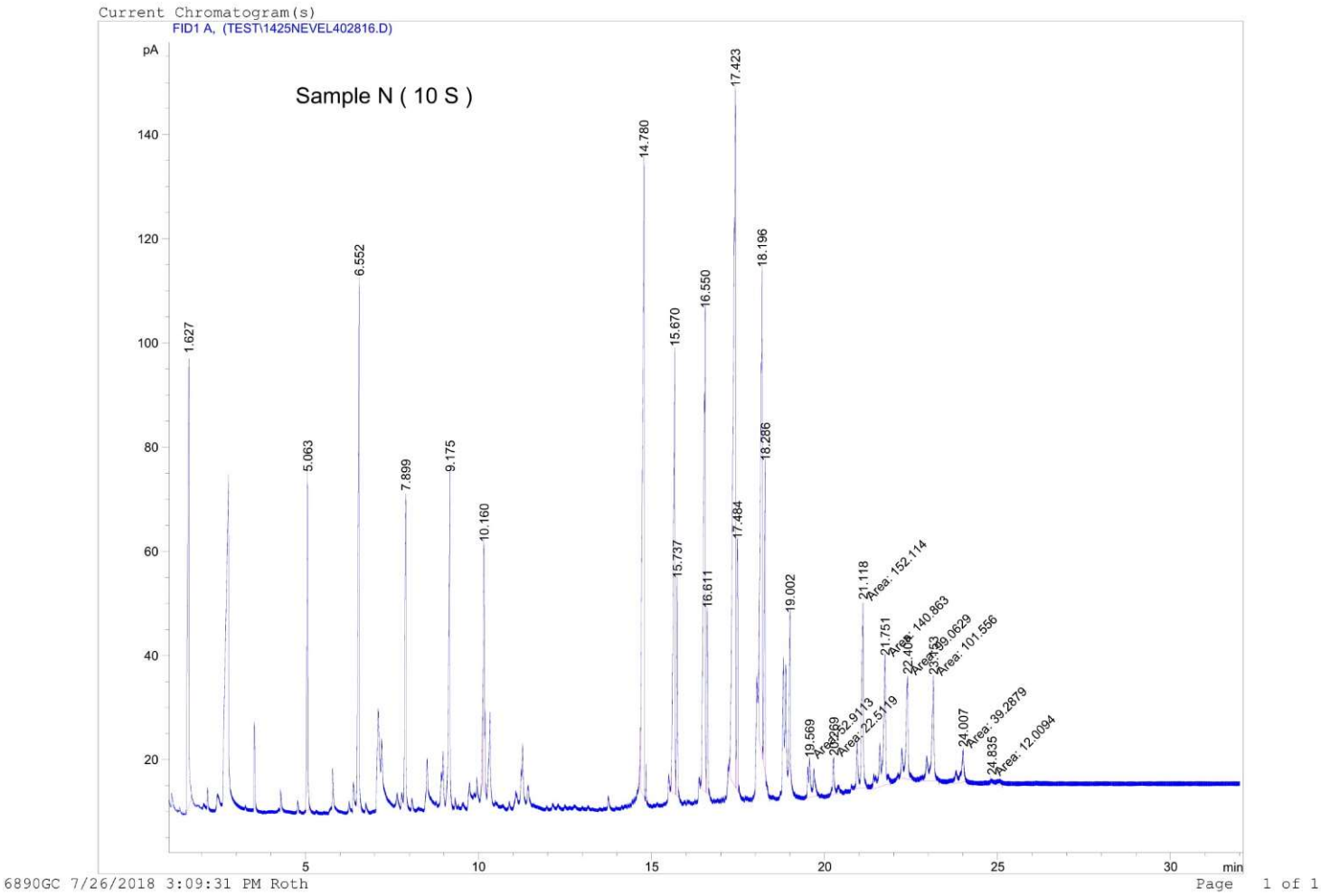

Fig. 6. Chromatogram of the wax ('N') with 10 % Radiacid 0407 (animal origin).

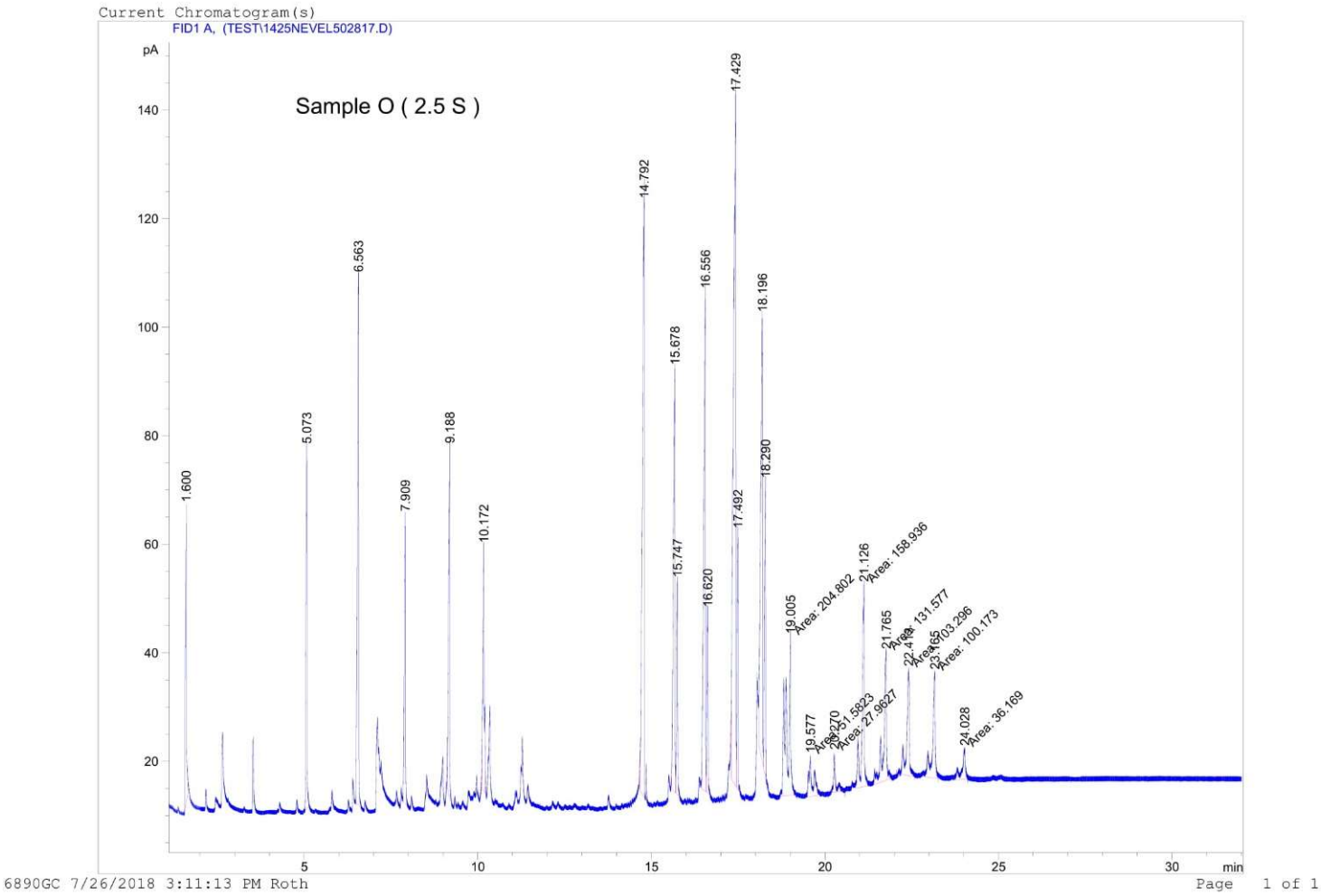

Fig. 7. Chromatogram of the wax ('O') with 2,5 % Radiacid 0464 (origin palm stearin).

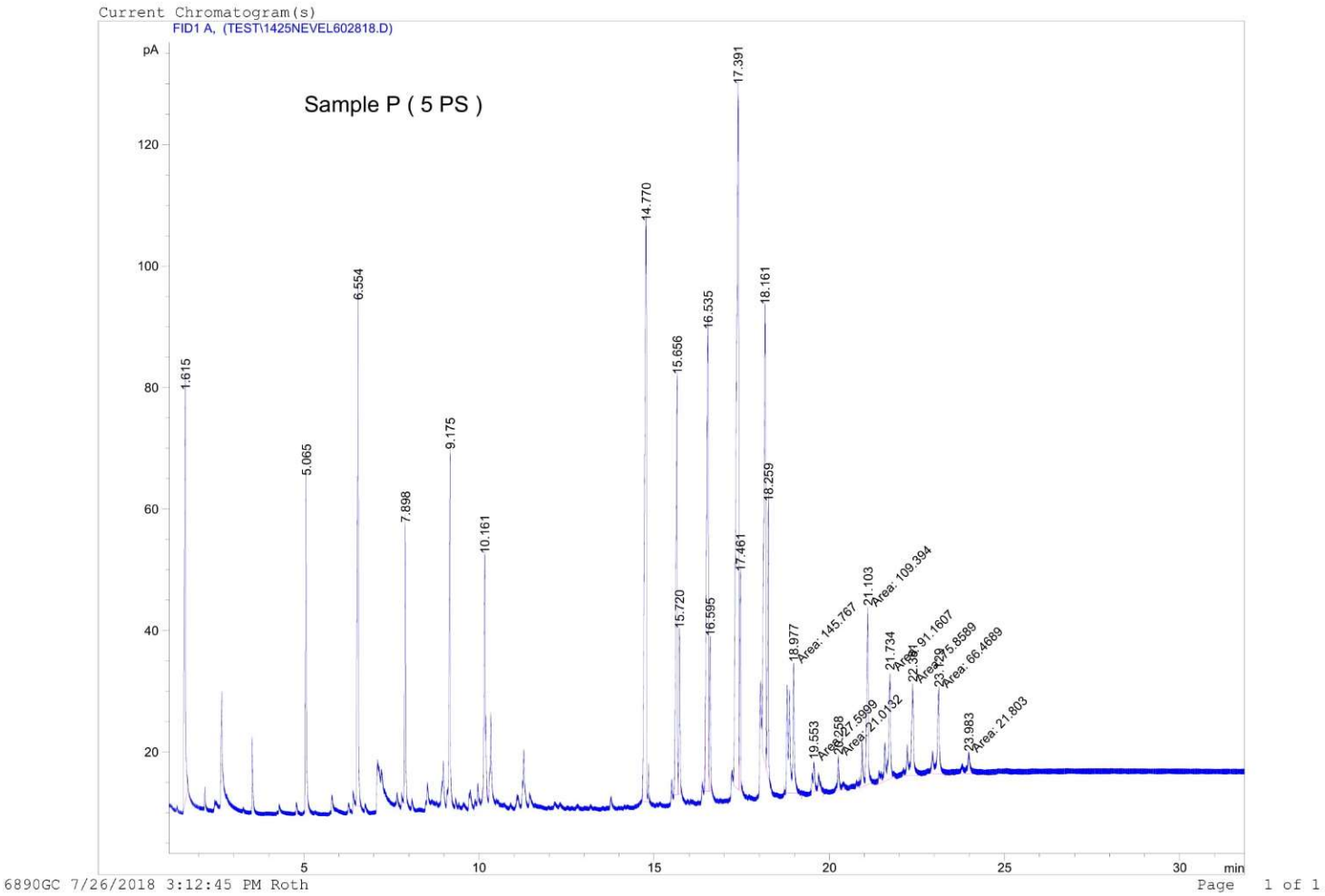

Fig. 8. Chromatogram of the wax ('P') with 5 % Radiacid 0464 (origin palm stearin).

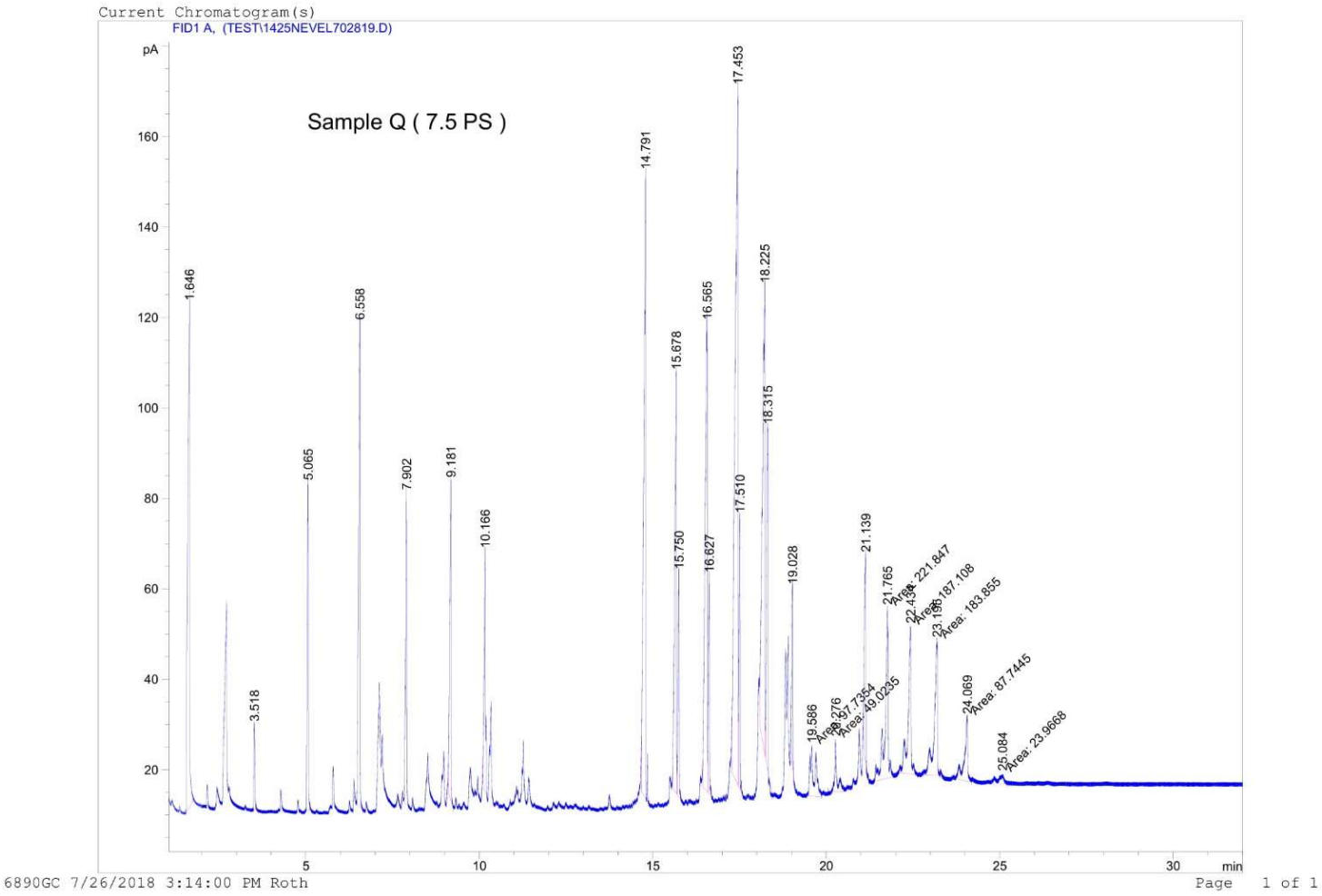

Fig. 9. Chromatogram of the wax ('P') with 7,5 % Radiacid 0464 (origin palm stearin).

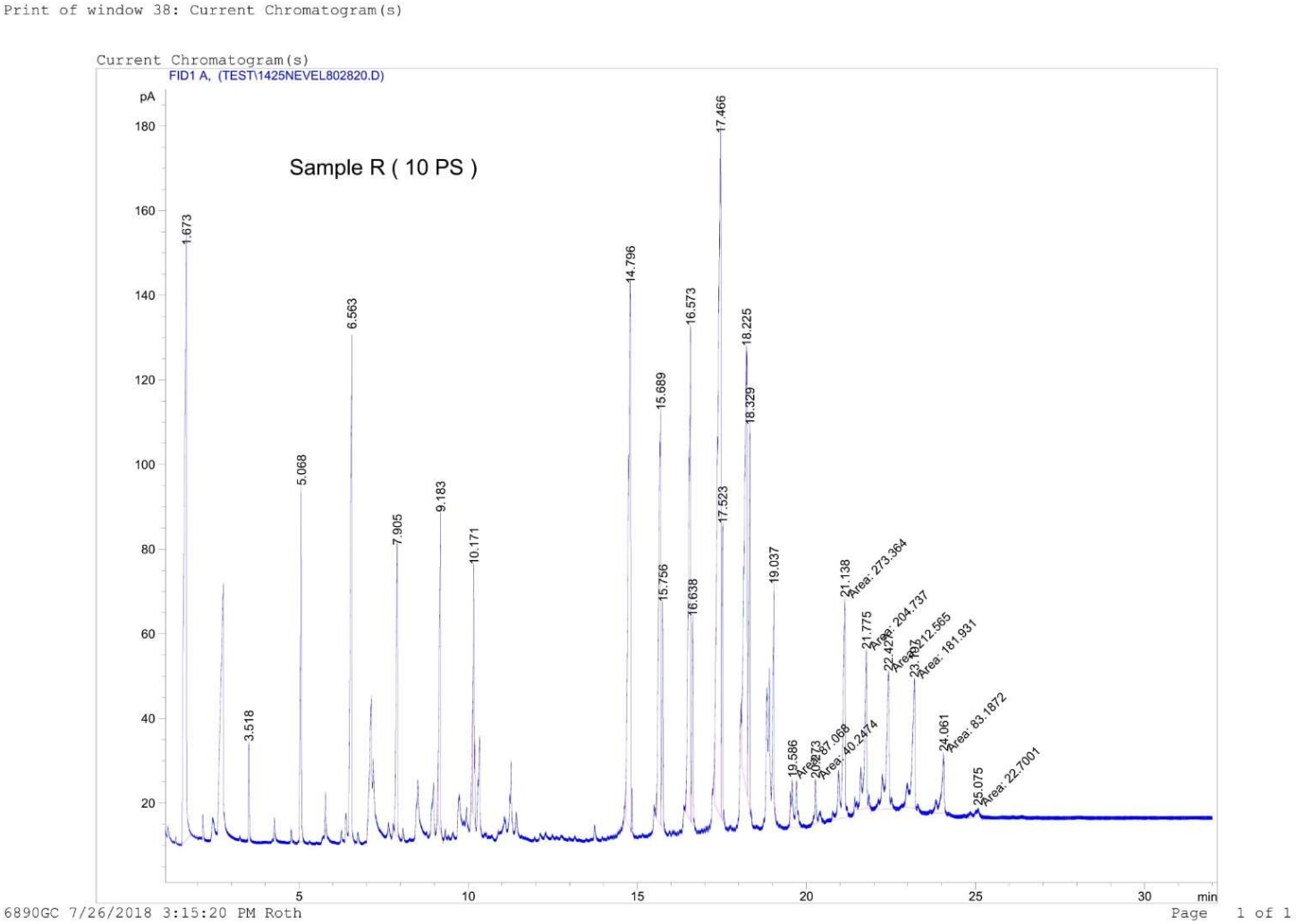

Fig. 10. Chromatogram of the wax ('O') with 10 % Radiacid 0464 (origin palm stearin).

Annex 2. Photographs

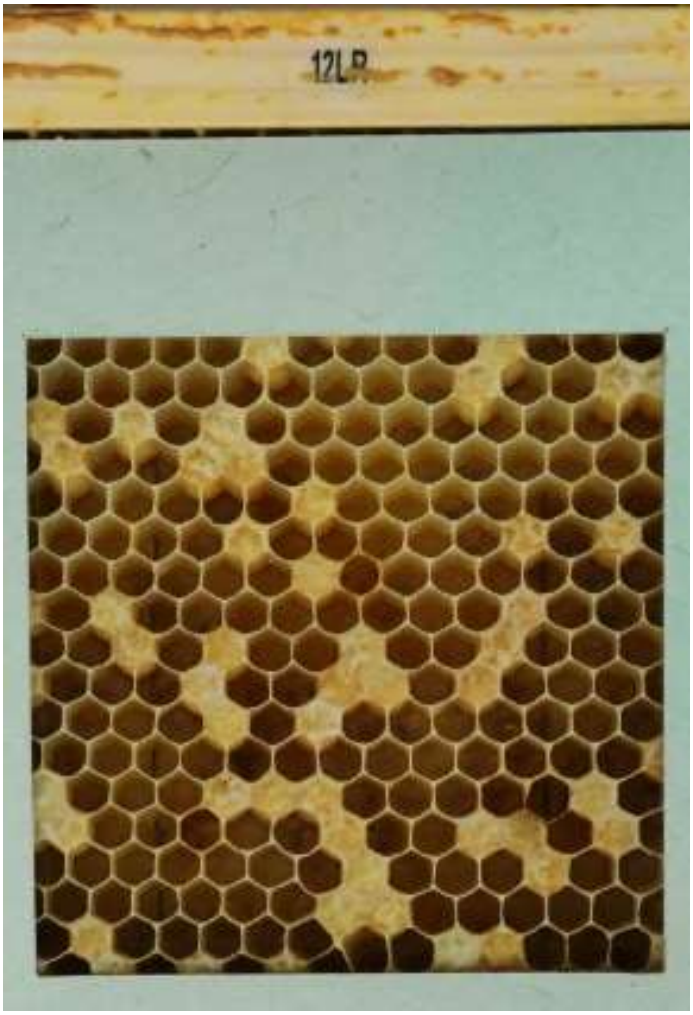

Foto 1. Detail of end phase for wax honeycomb ('R') (10 % Radiacid 0464) on test frame 12', left side as used during the counting of open and closed cells.

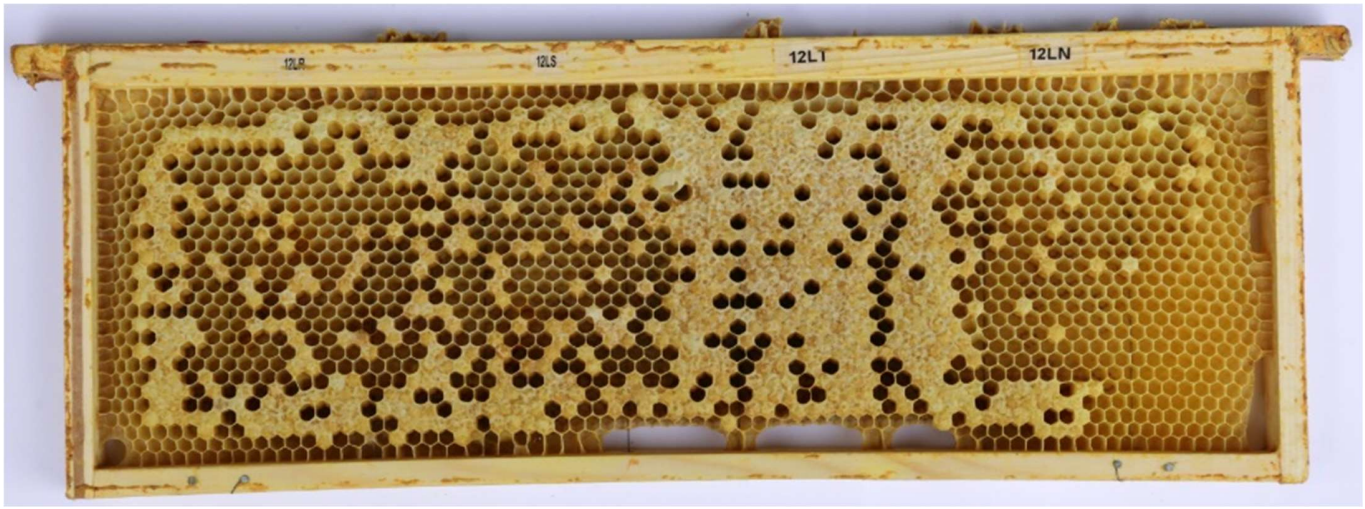

Foto 2. End phase test frame 12', left side.

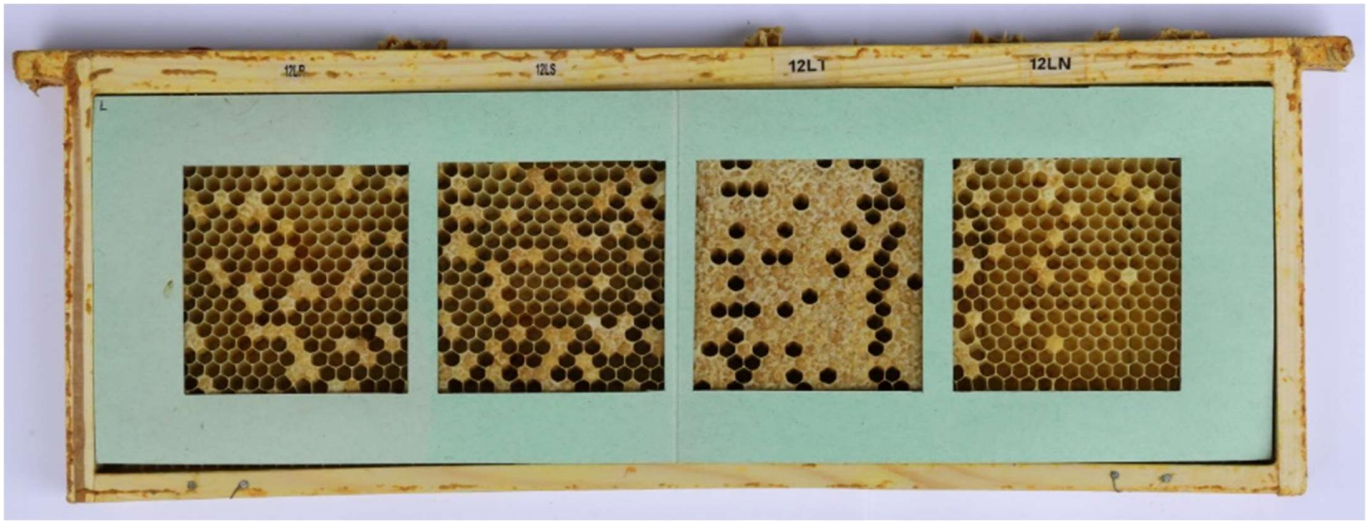

Foto 3. End phase test frame 12', left side, with template.