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(54) **Title:** METHOD FOR THE PREPARATION OF LIPID NANOPARTICLES

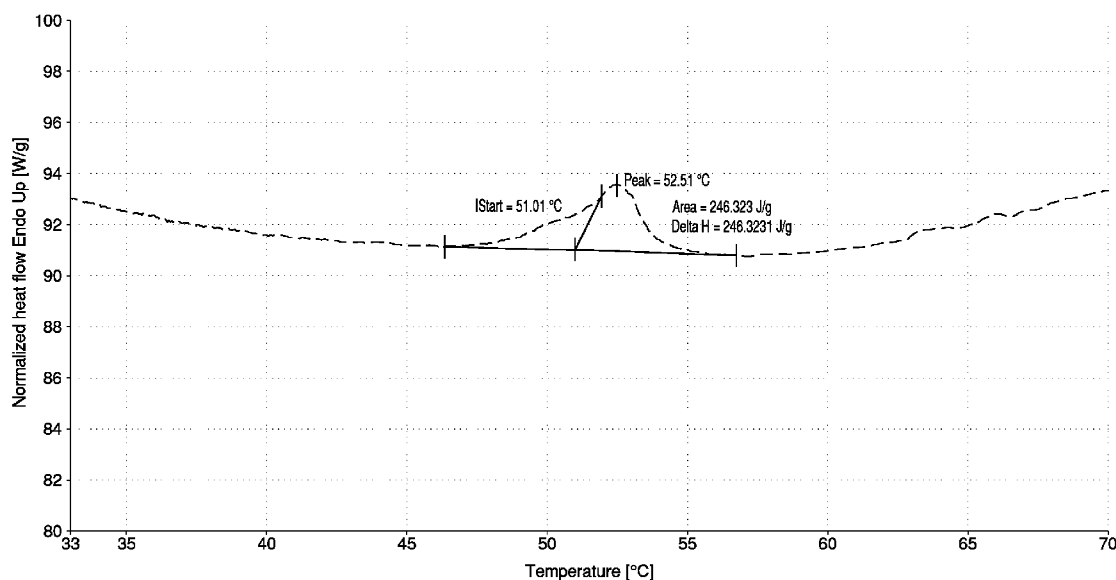

Fig.1

(57) **Abstract:** The method for the preparation of lipid nanoparticles, comprises: the phase of mixing in a single container a mixture comprising an aqueous solution with a solid lipid matrix and with at least one biocompatible non-ionic surfactant; the phase of heating the container to a working temperature of less than 100°C to obtain an emulsion; the phase of cooling the emulsion up to a temperature of less than 30°C to obtain solid lipid nanoparticles of a size of less than 500 nm.

WO 2020/254934 A1

[Continued on next page]

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METHOD FOR THE PREPARATION OF LIPID NANOPARTICLES

Technical Field

The present invention relates to a method for the preparation of lipid nanoparticles.

5 ## Background Art

The use of lipid nanoparticles in the pharmaceutical field for conveying pharmaceutical active ingredients is well known and widespread.

With particular reference to the pharmaceutical field, numerous methods for the preparation of lipid nanoparticles are known.

10 One of the most widely used techniques for the production of lipid nanoparticles is that of high pressure homogenization, which can be carried out either hot or cold. In both cases the drug is dissolved or solubilized in the melted lipid matrix (5-10°C above melting temperature). In the case of the hot homogenization technique, the resulting mixture is dispersed under agitation in 15 an aqueous surfactant solution, previously brought to the same temperature as the lipids. A pre-emulsion is thus obtained which is homogenized by means of high-pressure homogenizers and then cooled to crystallize the lipids and obtain solid lipid nanoparticles.

However, the hot technique cannot be used to incorporate into the nanoparticles 20 thermolabile drugs, which would be degraded due to the high temperatures, or hydrophilic drugs, which could break down in the aqueous phase during homogenization.

This method has been described in patent document EP0605497 and has many drawbacks, including the fact that it requires the use of complex and expensive 25 instruments, as well as the use of high temperatures.

In addition to this, the high cavitation forces generated determine the alteration of the conveyed active ingredient.

An alternative preparation method is based on the formation of hot micro-emulsions as precursors of the lipid nanoparticles.

30 Micro-emulsions are stable and transparent biphasic mixtures consisting of two immiscible liquids, water and oil, stabilized by a surfactant and a co-surfactant.

In particular, the size of the internal phase makes them suitable as precursors of nanoparticle systems.

This method, described in the patent document US1993/5250236, consists in the dilution of the micro-emulsion in cold water in order to break it and cause 5 the precipitation of the particles formed by the solidified lipids. However, even this method is not without its drawbacks.

In fact, one of the disadvantages of this method is related to the dilution of the initial micro-emulsion which results in obtaining a diluted suspension of nanoparticles, to which must be added the fact that this method is complex to 10 perform on a large scale.

In order to overcome at least part of these drawbacks, a method has been developed for the preparation of lipid nanoparticles, described in the patent document US2006/0292183, which makes it possible to obtain lipid nanoparticles by simple cooling of an oil micro-emulsion in water.

15 However, this method makes it possible to obtain a lipid concentration that can be used for the realization of an extremely low 0.2% w/v hot stable micro-emulsion and a cold nanoparticle suspension, greatly reducing the possibility of loading the active ingredient.

A further method of preparing lipid nanoparticles, described in patent document 20 WO 01/64328, consists in heating and cooling a mixture. This method is based on the principle of phase reversal, which requires the use of surfactants not approved by the Food and Drug Administration (FDA) and which therefore prevents their parenteral administration.

Description of the Invention

25 The main aim of the present invention is to provide a method for the preparation of lipid nanoparticles which makes it possible to prepare solid nanoparticles in concentrated aqueous solution stabilized by biocompatible surfactants and co-surfactants.

A further object of the present invention is to devise a method for the 30 preparation of lipid nanoparticles that allows considerably simplifying the preparation operations compared to methods of known type.

Another object of the present invention is to devise a method for the preparation of lipid nanoparticles that allows overcoming the above mentioned drawbacks of the prior art in a simple, rational, easy, effective to use and cost-effective solution.

5 Brief Description of the Drawings

Other characteristics and advantages of the present invention will be more evident from the description of a preferred, but not exclusive, embodiment of a method for the preparation of lipid nanoparticles, illustrated by way of an indicative, yet non-limiting example, in the attached tables of drawings 10 wherein:

Figure 1 is a graph drawn as a function of the thermal analysis of trimyristin nanoparticles produced with the method according to the present invention;

Figure 2 shows the comparison graph between the fractograms with UV detection of trimyristin 1, trimyristin 2 and trimyristin 3 produced by the 15 method according to the invention;

Figures 3-5 are representative graphs of the FFF DLS fractogram of the nanoparticles of trimyristin 1, trimyristin 2 and trimyristin 3, respectively;

Figures 6 and 7 are column graphs representative of the comparative analysis carried out with regard to the efficacy of incorporation and loading of the dye of 20 Trimyristin 1, Trimyristin 2 and Trimyristin 3, respectively, as a result of separation by gel filtration and separation by dextran centrifugation;

Figure 8 is a column chart of the cytotoxicity of trimyristin 2 lipid nanoparticles;

Figure 9-12 are column graphs of the comparative study on the biodistribution 25 of lipid nanoparticles marked with 6-coumarin in Wistar rats after intravenous administration.

Embodiments of the Invention

The present invention relates to a method for the preparation of solid lipid nanoparticles.

30 It is specified that the term “nanoparticles” in this treatise refers to particles with a size ranging from 1 nm to 100 nm.

According to the invention, the method involves the phase of mixing in a single container a mixture comprising an aqueous solution with a solid lipid matrix and with at least one biocompatible non-ionic surfactant.

Hereunder, the term "lipid matrix" relates to a matrix of lipophilic nature, insoluble in water and soluble in organic solvents, with low melting point and having a different chemical nature: paraffins, triglycerides, waxes, sterols, fatty acids, fatty alcohols.

The solid lipid matrix comprises at least one of either triglyceride, a mixture of aliphatic or sterol esters.

Preferably, the aforementioned mixture comprises hydrocarbons.

Advantageously, the surfactant is a polysorbate.

In detail, the surfactant is selected from the list comprising: Tween 20, Tween 40, Tween 60, Tween 80.

As an alternative, the surfactant is a derivative of sorbitan ethoxylate.

Furthermore, the mixture comprises a co-surfactant comprising an ester of sorbitan.

The co-surfactant is selected from the list comprising: Span 40, Span 60, Span 80.

The method comprises:

- the phase of heating the container to a working temperature of less than 100°C to obtain an emulsion;
- the phase of cooling the emulsion up to a temperature of less than 30°C to obtain solid lipid nanoparticles of a size of less than 500 nm.

The lipid matrix is present in a weight/volume concentration, evaluated with respect to the total weight of the aqueous solution, ranging from 0.3% to 5% and preferably from 0.5% to 4%.

Advantageously, the surfactant is present in a weight/volume concentration, evaluated with respect to the total weight of the aqueous solution, ranging from 1% to 20% and preferably from 2% to 16%.

Preferably, the co-surfactant is present in a weight/volume concentration, evaluated with respect to the total weight of the aqueous solution, ranging from

0.5% to 10%, preferably from 1% to 8%.

It should be pointed out that during the heating phase, the mixture is heated to a temperature higher than the lipid melting point and the cloud point of the surfactant, i.e. of the Tween, but lower than the boiling point of the water.

5 Under these conditions the Tween becomes insoluble in water, separates from the water solution of the same and interacts intimately with the surfactant, i.e. the Span, and with a melted lipid. This interaction takes place within a turbid-looking system in which the non-aqueous components of the mixture mix with each other.

10 Then, the method comprises a phase of purification of the solid lipid nanoparticles.

The phase of purification is selected from the group comprising: molecular exclusion chromatography, sedimentation and resuspension.

Advantageously, the solid lipid nanoparticles have a size ranging from 40 nm to 15 500 nm.

The present invention also relates to the use of solid lipid nanoparticles in formulations of radio-frequency drugs, anticancer drugs, radiopharmaceuticals, and in sensors.

In particular, these anticancer drugs are drugs for organs with a mass.

20 Examples of formulations of solid lipid nanoparticles are listed below.

EXAMPLE 1

Formulation of solid lipid nanoparticles of trimyristin and cholesteryl oleate.

The following table (Table 1) lists different formulations of solid lipid 25 nanoparticles obtained by means of the method according to the present invention and comprising a lipid selected from trimyristin, cholesteryl palmitate or tripalmitin.

LIPID NANOPARTICLES	Lipid (mg)	Span 80 (µl)	Tween 20 (µl)	De-ionized water (ml)
Trimyristin 1	100	200	400	5
Trimyristin 2	100	200	300	5

Trimyristin 3	100	200	250	5
Cholesteryl palmitate 1	100	200	400	5
Cholesteryl palmitate 2	100	200	300	5
Tripalmitin	100	200	300	5

Table 1

The lipid selected from Trimyristin and Cholesteryl palmitate was mixed with the aqueous solution, Tween 20 and Span 80. This solution was heated to 80°C, i.e. a temperature above the cloud point of Tween 20.

5 Subsequently this solution was cooled to 60°C, reaching this temperature permits the formation of a micro-emulsion.

It is specified that, in this treatise, the term “micro-emulsion” relates to a biphasic stable and transparent colored mixture, consisting of two immiscible liquids (water and oil) stabilized by a surfactant and generally by a co-

10 surfactant.

In detail, the micro-emulsion is clear and the lipid nanoparticles are suspended therein.

Afterwards, the micro-emulsion is cooled down to room temperature, resulting in the precipitation of nanoparticles.

15

EXAMPLE 2

Thermal analysis of the trimyristin 2 nanoparticles

The thermal analysis was conducted by differential scanning calorimetry (DSC) on the nanoparticles in suspension both before and after the purification phase.

20 In detail, the purification phase was performed by molecular exclusion (gel filtration technique with exclusion limit of 100000 Da).

In particular, the gel filtration removes the excess Tween 20, free and in micelles.

As can be seen in Figure 1, before purification, the micro-emulsion shows no

25 phase transitions in light of the fact that the lipid melting temperature falls within the temperature range of existence and stability of the micro-emulsion.

Instead, the sample purified from micelles shows a melting transition typical of trimyristin.

EXAMPLE 3

5 Dimensional analysis of the trimyristin nanoparticles

The dimensional analysis of the trimyristin nanoparticles (Trimyristin 1, Trimyristin 2, Trimyristin 3) was conducted by means of Dynamic Light Scattering (DLS).

The table below (Table 2) shows the results obtained:

Lipid nanoparticles	Average diameter (nm)	Polydispersion
Trimyristin 1	158.1	0.140
Trimyristin 2	251.4	0.019
Trimyristin 3	424.3	0.157

10 Table 2

Dimensional analysis was further detailed with Field Flow Fractionation (FFF) with UV and DLS detection.

Figure 2 shows the comparison graph between the fractograms with UV detection of trimyristin 1, trimyristin 2 and trimyristin 3.

15 The peaks corresponding to Trimyristin 1, Trimyristin 2 and Trimyristin 3 were further analyzed with DLS detection.

The Figures 3, 4 and 5 show the comparative graph of the fractograms obtained with FFF UV.

In particular, Figure 3 shows the FFF DLS fractogram of the nanoparticles of

20 trimyristin 1 characterized by:

- peak 1: 38 nm;
- peak 2: 70 nm;
- peak 3: 136 nm.

Likewise, Figure 4 shows the FFF DLS fractogram of the nanoparticles of

25 trimyristin 2 characterized by:

- peak 1: 59 nm;
- peak 2: 202 nm.

Again, Figure 5 shows the FFF DLS fractogram of the nanoparticles of

trimyristin 3 characterized by:

- peak 1: 72 nm;
- peak 2: 407 nm.

The analysis of these graphs shows that the trimyristin 1 nanoparticles, which have a higher polydispersion than the DLS batch reading, are composed of a mixture of three populations. Those of trimyristin 2 and 3, on the other hand, are made up of a majority population, with a limited quantity of smaller nanoparticles.

The average size of the trimyristin 1 nanoparticles was further analyzed by DLS, after being purified with molecular exclusion techniques (gel filtration and gel centrifugation) and then sedimentation (centrifugation after 1:1 dilution with 30% dextran, followed by resuspension of the precipitate in water). The gel filtration has an exclusion limit of 100000 Da; this means that this type of technique excludes the Tween 20 in monomer form and in micelles.

Moreover, sedimentation by centrifugation separates the nanoparticles from all the hydrosoluble components.

Just prepared		Centrifugated gel		Filtered gel		Centrifugated in dextran and resuspended	
Average dimension (nm)	polydispersion	Average dimension (nm)	polydispersion	Average dimension (nm)	polydispersion	Average dimension (nm)	polydispersion
158.1	0.140	158.9	0.153	160.3	0.135	176.7	0.074

Table 3

EXAMPLE 4

Loading and incorporation of dyes in the nanoparticles of trimyristin, tripalmitin and cholesteryl palmitate.

The lipid nanoparticles were loaded with two lipophilic fluorescent dyes: Nile red and 6-coumarin.

These dyes were added to the container prior to heating.

The excess dye was subsequently removed following spontaneous sedimentation.

The analysis of the incorporation took into account two parameters:

- loading capacity (i.e. the ratio between the conveyed dye and the lipid);
- incorporation efficiency (i.e. the ratio between the dye incorporated in the lipid matrix and the total dye present in the formulation).

5 In detail, the techniques for measuring the dye incorporation efficiency in the nanoparticles are: molecular exclusion techniques (gel filtration, gel centrifugation) and sedimentation.

In order to evaluate the incorporation efficiency, the formulations of trimyristin 1, trimyristin 2 and trimyristin 3 loaded with 6-coumarin and Nile red were 10 analyzed.

As can be seen in Figure 6, separation by gel centrifugation resulted in an incorporation efficiency greater than 90%.

The results obtained by gel filtration and sedimentation are shown in Figures 6 and 7. In particular, the loading capacity was calculated following 30% dextran 15 centrifugation and resuspension of the trimyristin and cholesteryl palmitate nanoparticles loaded with 6-coumarin.

Subsequently, 100 microliters of the suspended lipid nanoparticles were taken and subsequently diluted in 900 microliters of ethanol in order to extract the dye. The results obtained are shown below (Table 4).

Lipid nanoparticles	Mg dye/g lipid
Trimyristin 1	1.20
Trimyristin 2	1.35
Trimyristin 3	1.75
Cholesteryl palmitate 1	72
Cholesteryl palmitate 2	138

20 Table 4

From the above data, it is evident that the Cholesteryl palmitate shows a surprisingly higher loading capacity than trimyristin.

EXAMPLE 5

25 Cytotoxicity of the trimyristin 2 nanoparticles.

Cytotoxicity was studied on tumor cell lines with the MTT assay after 72 hours

of incubation (Figure 8).

In fact, despite the excipients used for the preparation of the nanoparticles being safe and biocompatible, surfactants may cause cytotoxicity after long exposure on cells. Therefore, the effect of the surfactant purification method (Tween 20) 5 on cytotoxicity after 72 hours of exposure was considered. Purification was performed using the methods described in example 3.

As can be seen in Figure 8, the purification technique considerably affects the cytotoxicity of lipid nanoparticles.

Gel filtration is in fact able to ensure the lowest cytotoxicity. Purification by 10 centrifugation with 30% dextran and resuspension makes it possible to minimize cytotoxicity only after 1:200 dilution of the sample in culture medium.

This suggests that the gel filtration method is the most effective in removing the surfactant. On the contrary, gel centrifugation does not seem to work due to the 15 molecular exclusion range used (6000 Da), which does not permit separating surfactant micelles from the nanoparticles.

EXAMPLE 6

Biodistribution of lipid nanoparticles marked with 6-coumarin in Wistar rats 20 (250g) after intravenous administration.

The following formulations were used (Figures 9-12):

Solid lipid nanoparticles	SLN 1	SLN2	SLN3	SLN4
Cholesteryl palmitate (mg)	100	100	30	100
Tripalmitin (mg)			70	
Span 80 (μl)	200	200	200	200
Tween 20 (μl)	350	375	375	
Tween 80 (μl)				500
Water (ml)	5	5	5	5
Purification	Centrifugation in 30% dextran	Gel filtration (dilution 1:2)	Gel filtration (dilution 1:2)	Gel filtration (dilution 1:2)
Average	170.1	148.1	246.5	221.8

dimension (nm)				
Polydispersion	0.130	0.124	0.105	0.227

Table 5

The tissues were homogenized 1:4 with water and the blood centrifuged at 4000 rpm. Plasma and homogenate were diluted 1:4 with methanol and centrifuged. The supernatant was then injected into an HPLC.

5 There seems to be no difference in the biodistribution of nanoparticles formulated with different lipids or surfactants. There seems to be an influence of the method of purification of nanoparticles on their biodistribution: particles purified with 30% dextran show a significant increase in accumulation at brain level; this can be put down to the higher concentration of residual surfactant

10 present in suspension.

It has in practice been ascertained that the described invention achieves the intended objects.

In particular, the fact is underlined that the particular solution of providing a mixture comprising an aqueous solution with a solid lipid matrix and at least

15 one non-ionic biocompatible surfactant inserted in a single container, permits obtaining concentrated solid lipid nanoparticles, avoiding dilutions.

CLAIMS

1) Method for the preparation of lipid nanoparticles, comprising:

- the phase of mixing in a single container a mixture comprising an aqueous solution with a solid lipid matrix and with at least one biocompatible non-
5 ionic surfactant;
- the phase of heating said container to a working temperature of less than 100°C to obtain an emulsion;
- the phase of cooling said emulsion up to a temperature of less than 30°C to obtain solid lipid nanoparticles of a size of less than 500 nm.

10 2) Method according to claim 1, characterized by the fact that said solid lipid matrix comprises at least one of triglyceride, a mixture of saturated aliphatic hydrocarbons and esters.

3) Method according to one or more of the preceding claims, characterized by the fact that said solid lipid matrix comprises hydrocarbons.

15 4) Method according to one or more of the preceding claims, characterized by the fact that said surfactant is a polysorbate.

5) Method according to one or more of the preceding claims, characterized by the fact that said surfactant is a derivative of sorbitan ethoxylate.

6) Method according to one or more of the preceding claims, characterized by
20 the fact that said mixture comprises a co-surfactant comprising an ester of sorbitan.

7) Method according to one or more of the preceding claims, characterized by the fact that said lipid matrix is present in a weight/volume percentage concentration, evaluated with respect to the total weight of the aqueous solution,
25 ranging from 0.3% to 5% and preferably from 0.5% to 4%.

8) Method according to one or more of the preceding claims, characterized by the fact that said surfactant is present in a weight/volume percentage concentration, evaluated with respect to the total weight of the aqueous solution, ranging from 1% to 20% and preferably from 2% to 16%.

30 9) Method according to one or more of the preceding claims, characterized by the fact that said co-surfactant is present in a weight/volume percentage

concentration, evaluated with respect to the total weight of the aqueous solution, ranging from 0.5% to 10%, preferably from 1% to 8%.

10) Method according to one or more of the preceding claims, characterized by the fact that it comprises a phase of purification of said solid lipid nanoparticles.

5 11) Method according to one or more of the preceding claims, characterized by the fact that said phase of purification is selected from the group comprising: molecular exclusion chromatography, sedimentation and resuspension.

12) Method according to one or more of the preceding claims, characterized by the fact that said solid lipid nanoparticles have a size ranging from 40 nm to 500

10 nm.

13) Use of lipid nanoparticles obtained according to one or more of the preceding claims, in formulations of radio-frequency drugs, anticancer drugs, radiopharmaceuticals, and in sensors.

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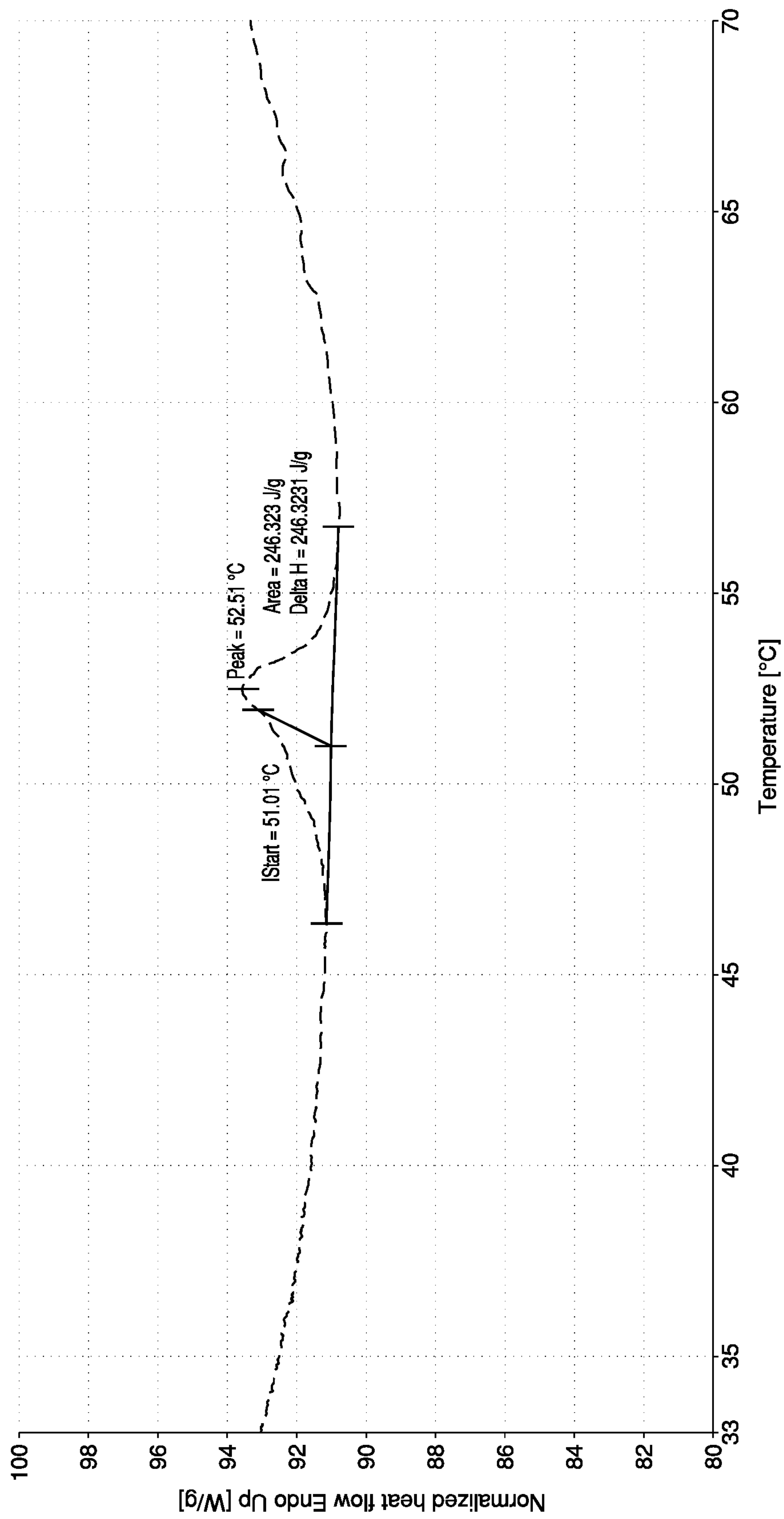

Fig.1

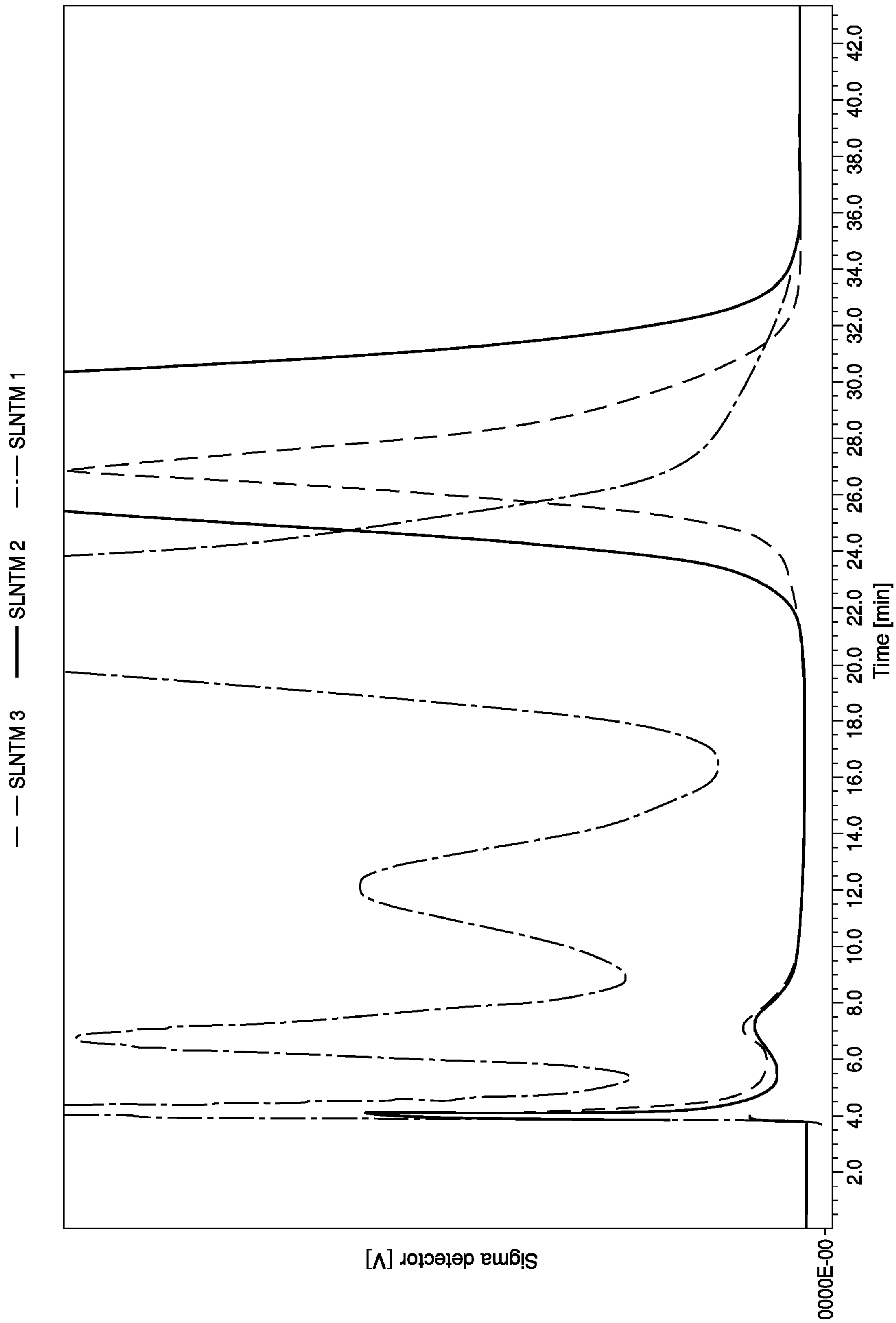

Fig.2

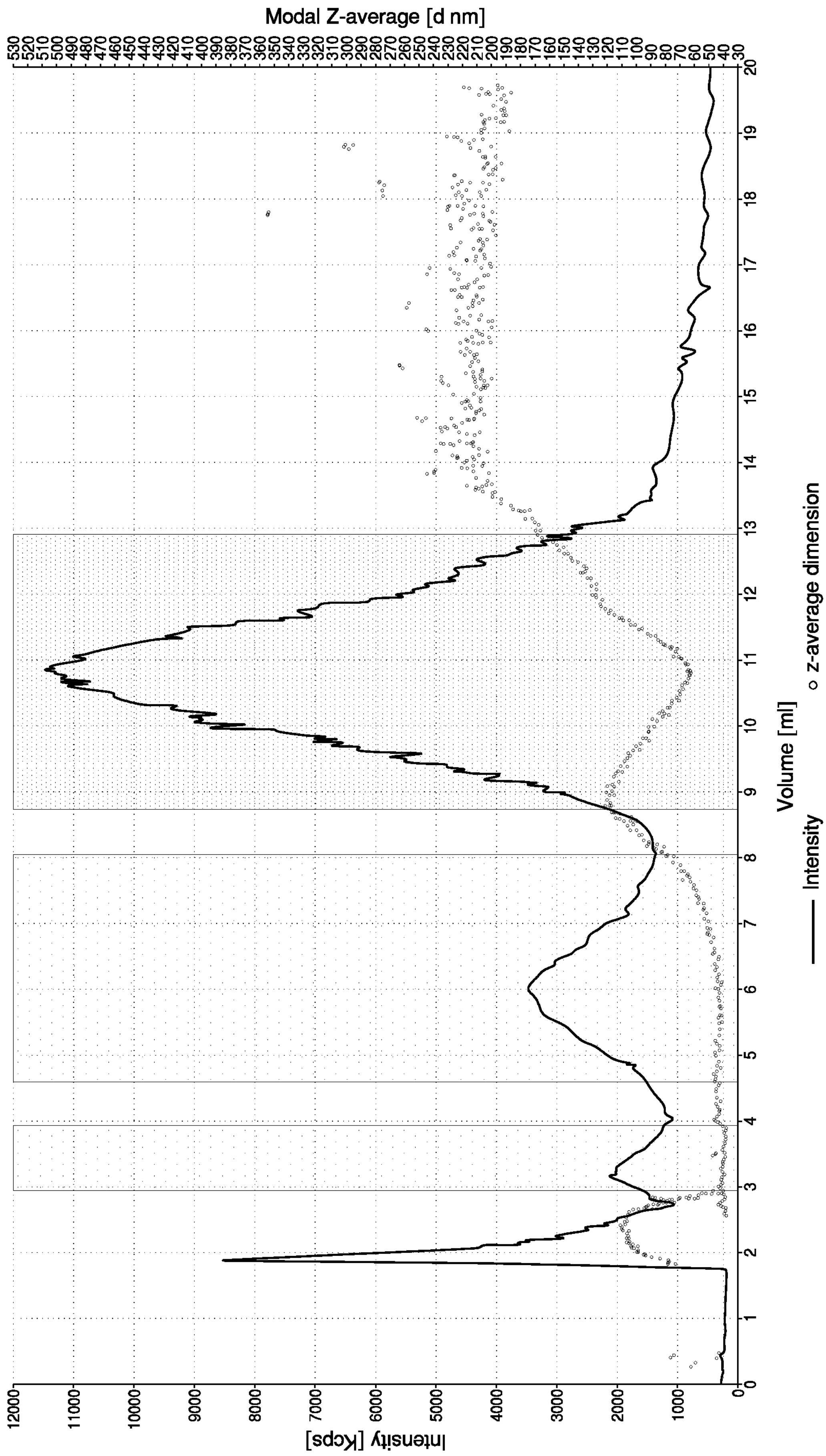

Fig.3

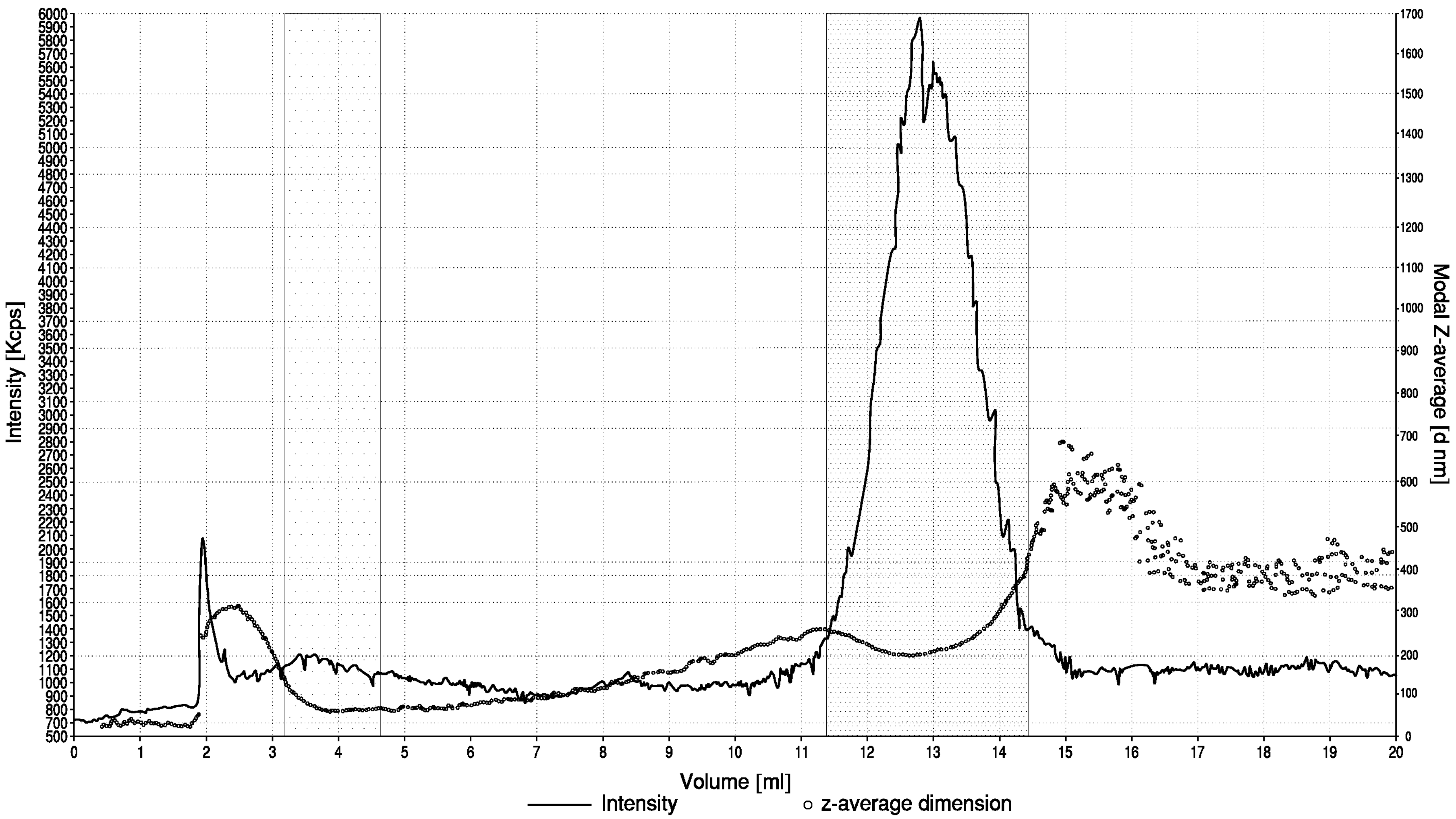

Fig.4

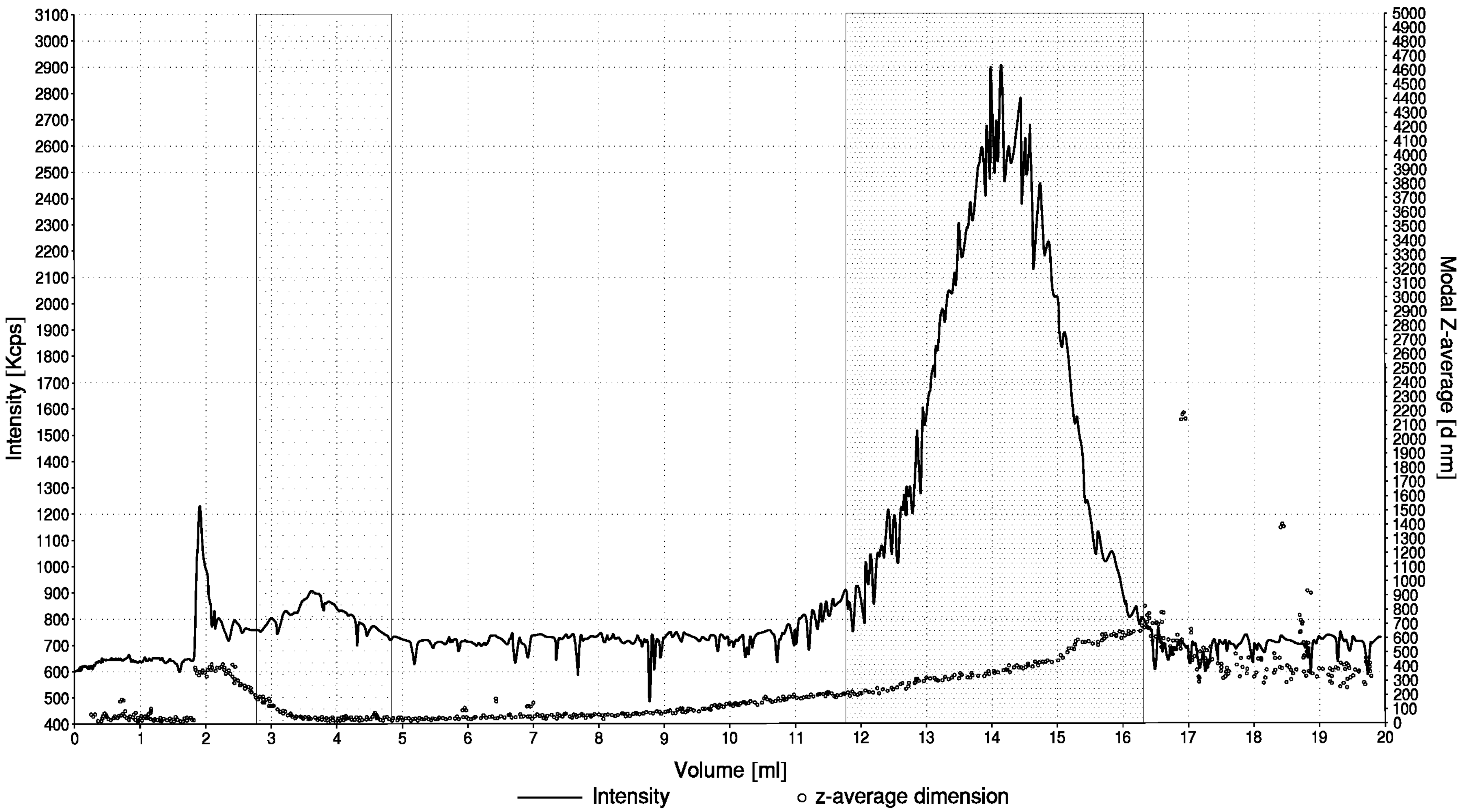

Fig.5

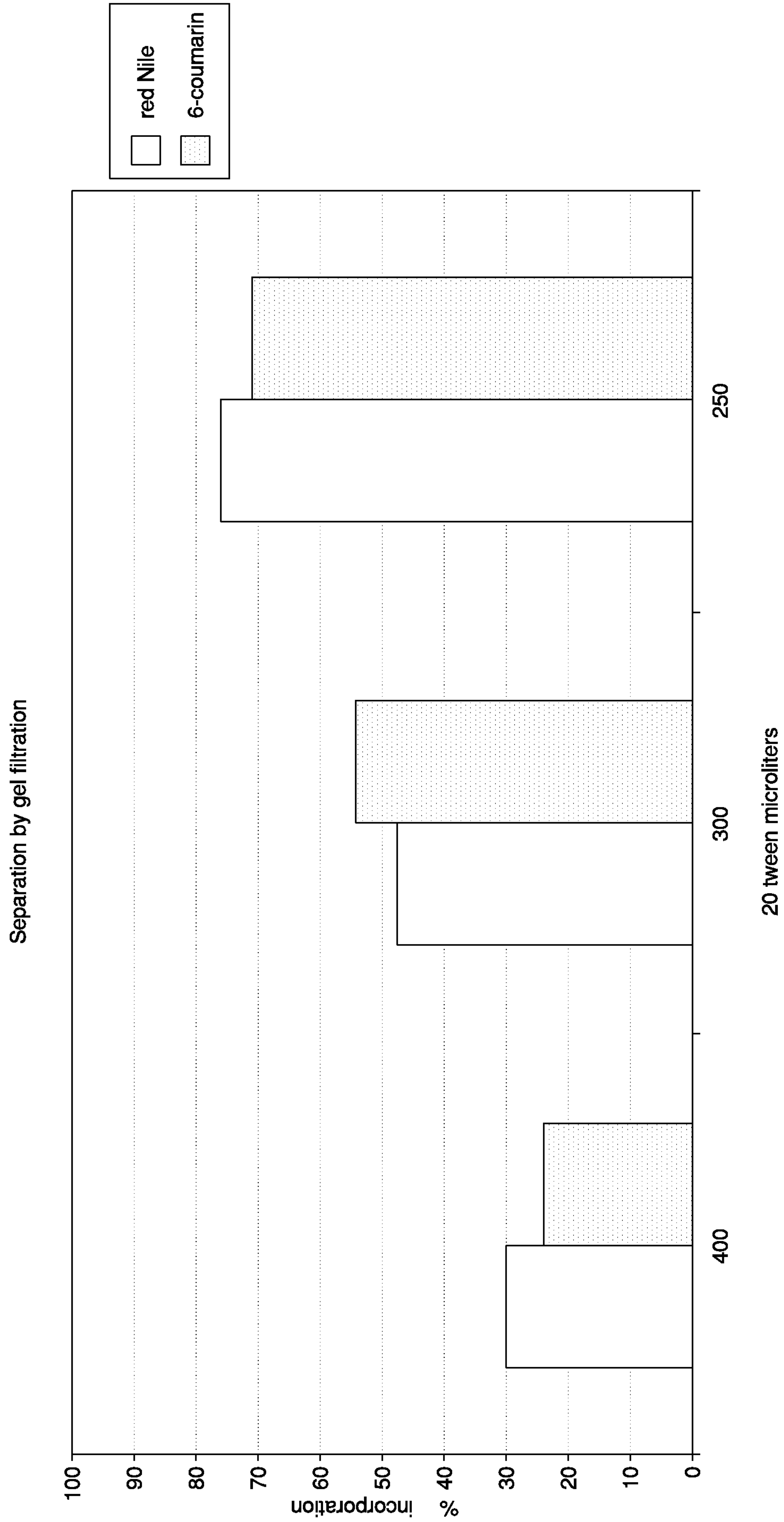

Fig.6

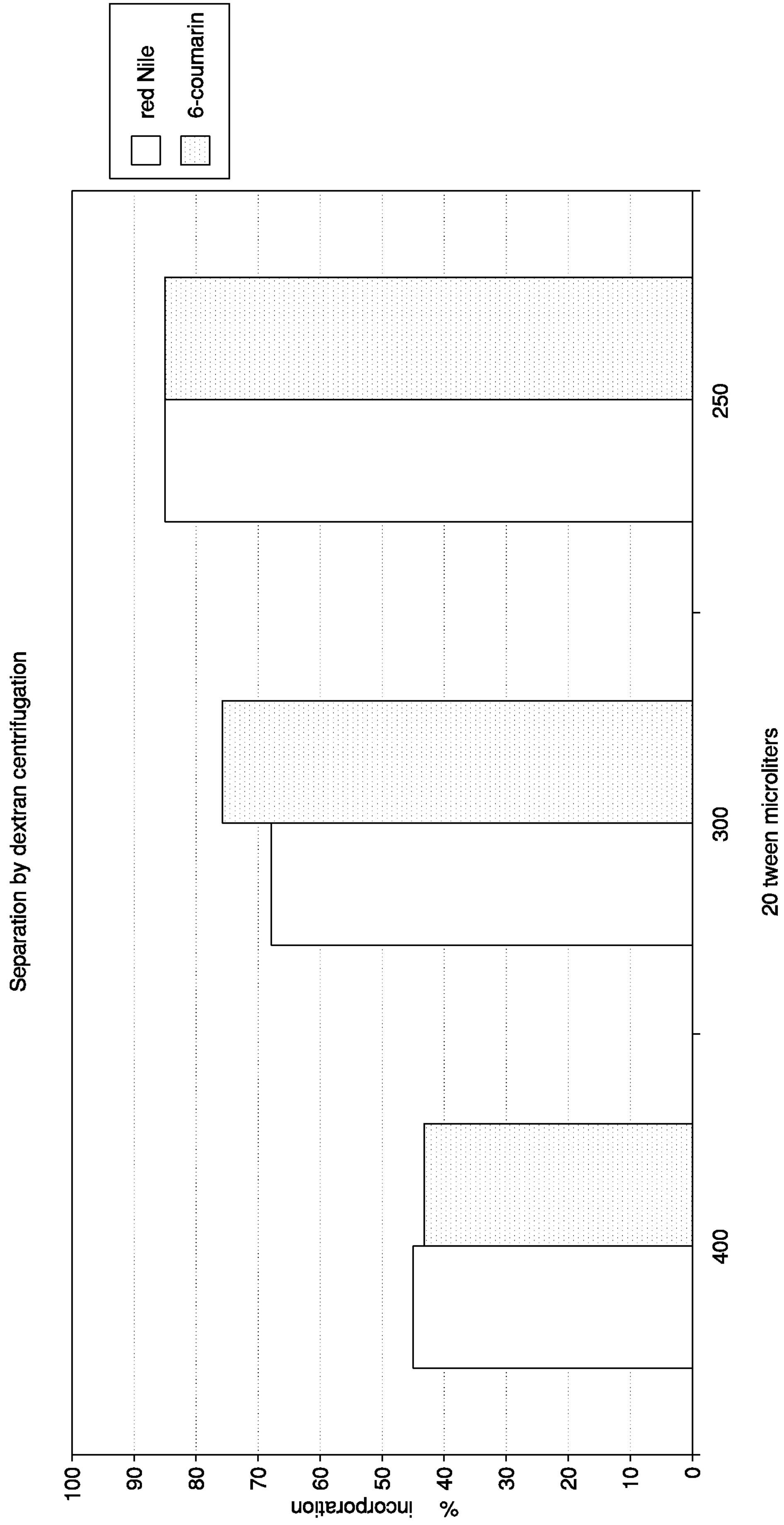

Fig.7

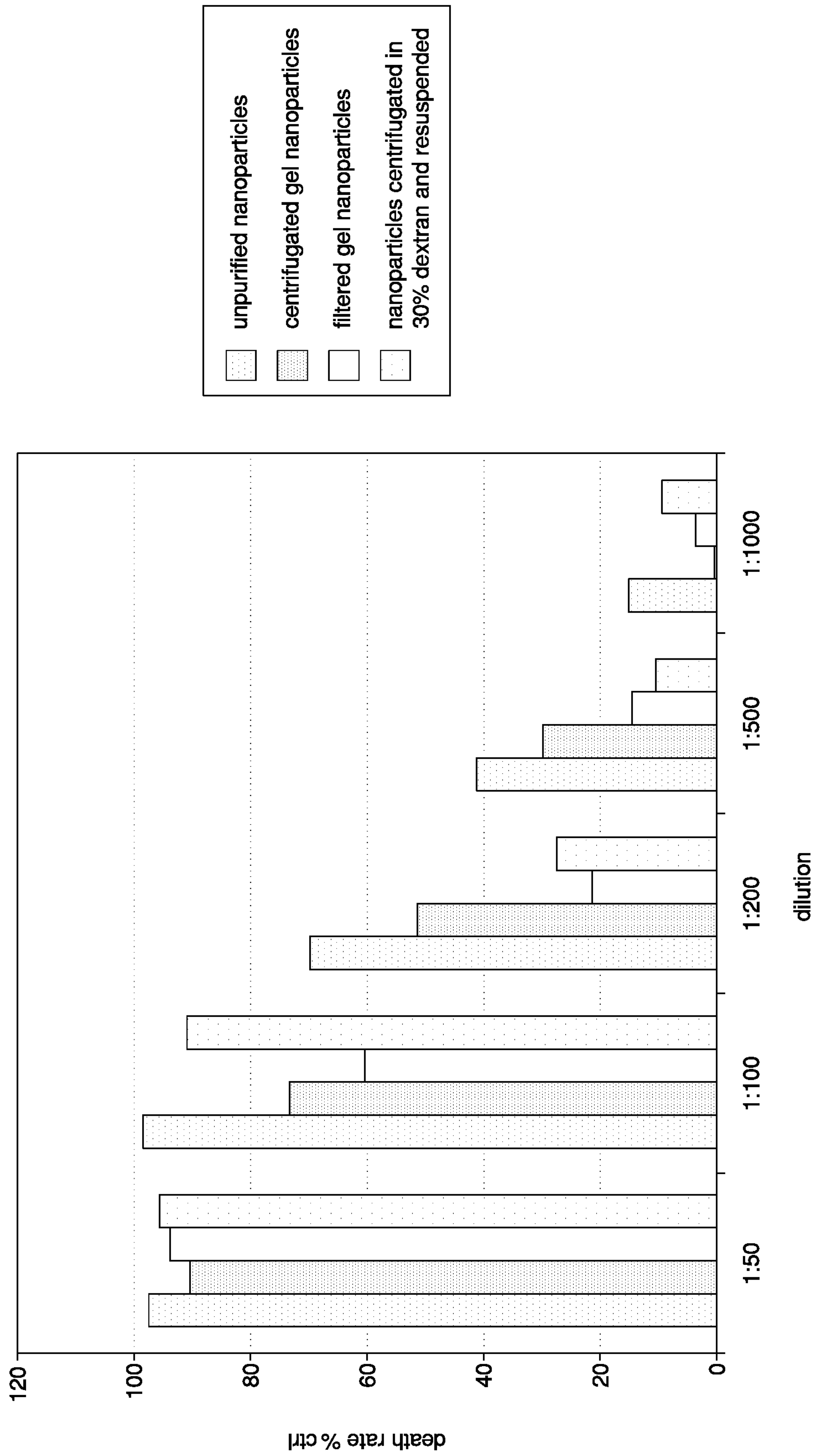

Fig.8

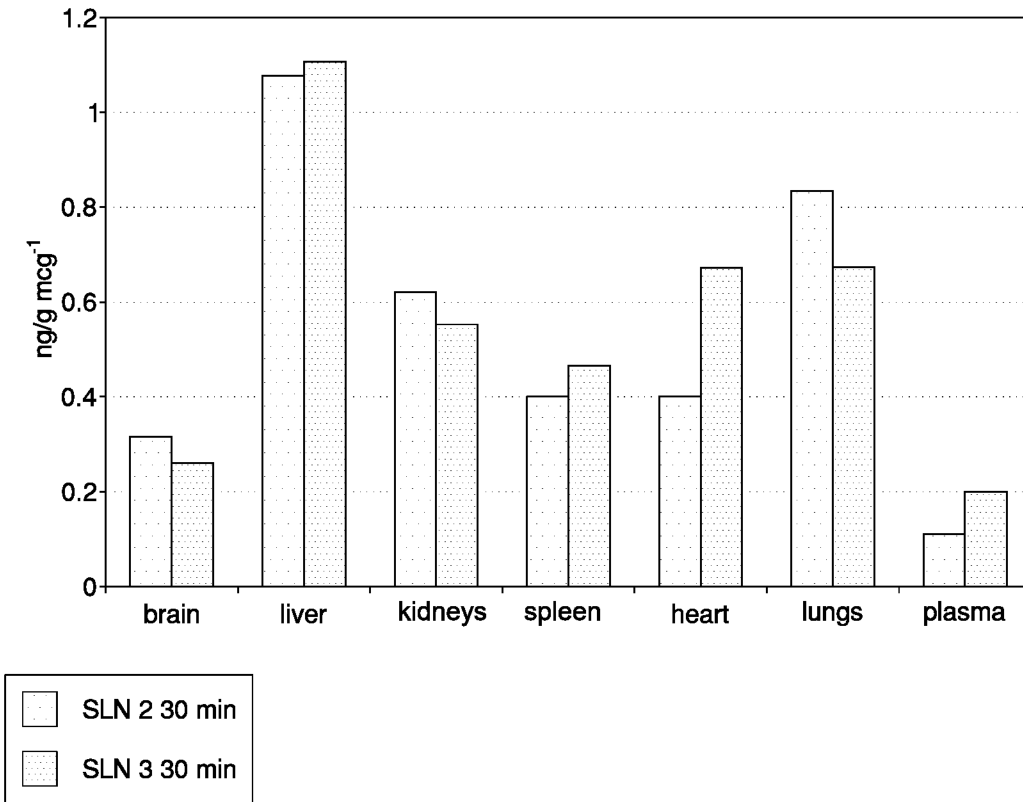

Fig.9

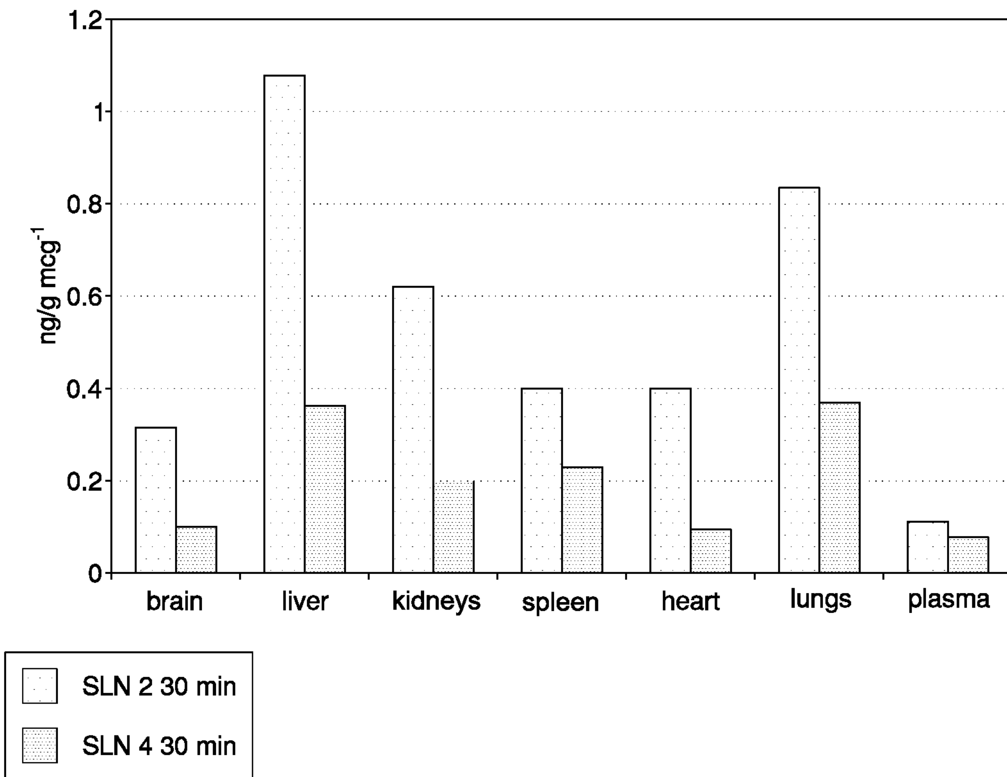

Fig.10

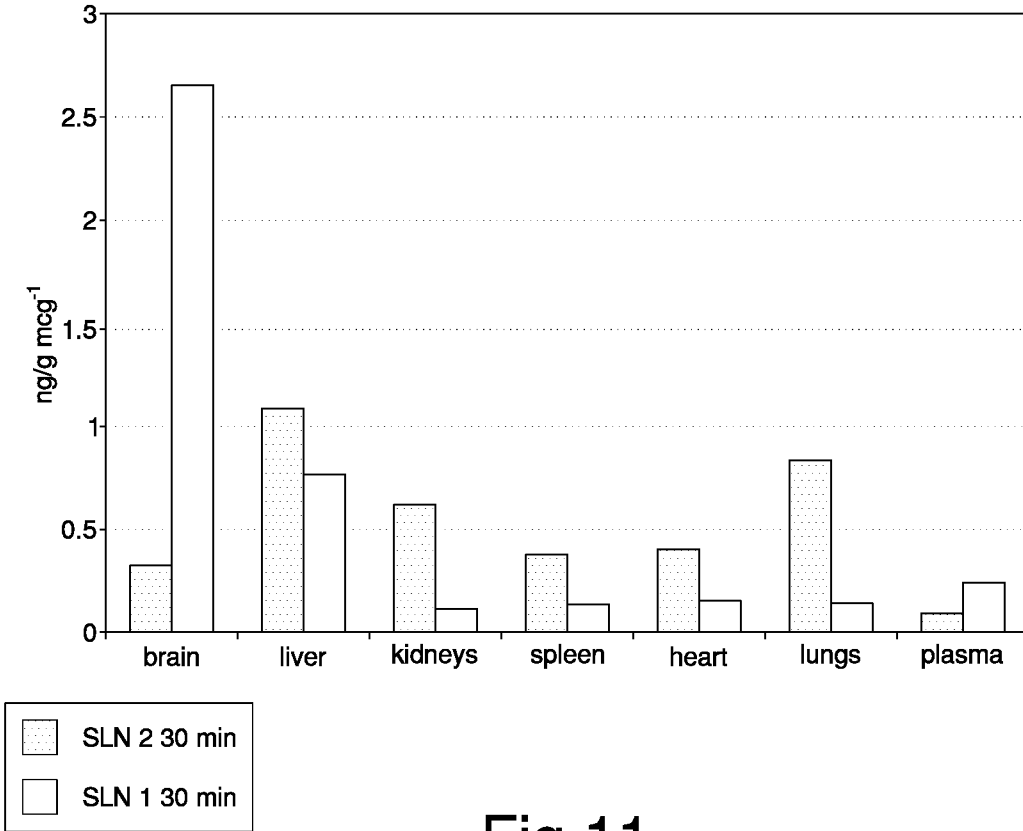

Fig.11

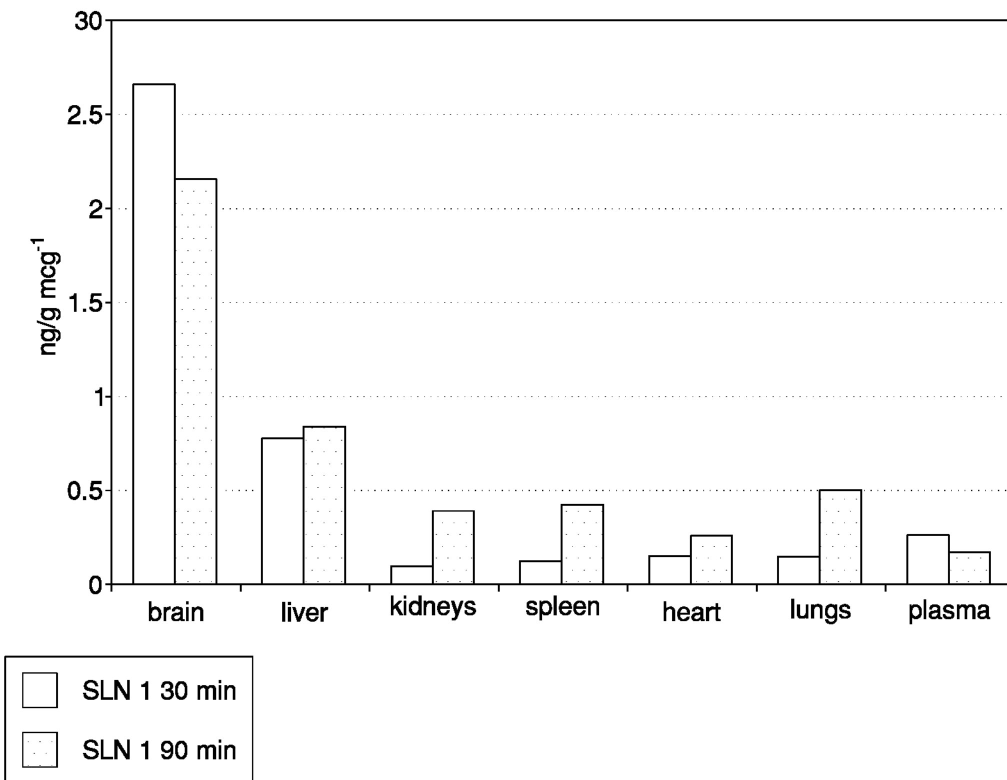

Fig.12

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2020/055562

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K9/51 A61B5/00 A61K31/00 A61K49/00 A61K51/00
B01J13/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61B B01J

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SHAH ROHAN M ET AL: "Physicochemical characterization of solid lipid nanoparticles (SLNs) prepared by a novel microemulsion technique", JOURNAL OF COLLOID AND INTERFACE SCIENCE, ACADEMIC PRESS,INC, US, vol. 428, 4 May 2014 (2014-05-04), pages 286-294, XP028850061, ISSN: 0021-9797, DOI: 10.1016/J.JCIS.2014.04.057 page 287; table 1 Par. 2.2.2.2., Par. 2.2.4.1.; page 288 Par. 3.2.; page 289 Par. 3.3.; page 290; figure 2 -----	1,3-8, 10-13

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
7 October 2020	22/10/2020

Name and mailing address of the ISA/	Authorized officer
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INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2020/055562

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2018/109690 A1 (UNIV DO PORTO [PT]; UNIV FEDERAL DE PERNAMBUCO UFPE [BR]) 21 June 2018 (2018-06-21) paragraph [0144] - paragraph [0152] paragraph [0153] - paragraph [0159] paragraph [0160] - paragraph [0169] paragraph [0170] - paragraph [0177] paragraph [0184] - paragraph [0191] paragraph [0192] - paragraph [0198] paragraph [0199] - paragraph [0206] paragraph [0215] - paragraph [0222] paragraph [0223] - paragraph [0232] paragraph [0248] - paragraph [0257] paragraph [0263] paragraph [0264] paragraph [0266] paragraph [0272] - paragraph [0280] paragraph [0272] - paragraph [0276] paragraph [0281] - paragraph [0290] paragraph [0309] - paragraph [0315] paragraph [0045] paragraph [0072] - paragraph [0075] paragraph [0087] paragraph [0089] paragraph [0091] -----	1,2,4, 7-9,12, 13

INTERNATIONAL SEARCH REPORT

Information on patent family members

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2018109690 A1	21-06-2018	NONE	