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Reference Substances
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Certificate of analysis

Article:	89871 Escin
Certificate # / Lot Number:	78379
Material batch:	9758
Sample-ID:	41002
End of analysis:	10/2020
Expiry date:	08/2028

Test	Unit	Specified value	Testresult
Appearance, SOP 100005		powder	conform
Color, SOP 100006		white	conform
Identification (UV spectrum from HPLC-DAD analysis) according to specification, SOP 204311		conform	conform
Identification (1H-NMR-spectroscopy), (outsourced), SOP 206010		conform	conform
Identification (13C-NMR-spectroscopy), (outsourced), SOP 206020		conform	conform
Identification (IR-spectroscopy, Ph.Eur. 10.3, 2.2.24 / USP43 NF37 <197>), SOP 206000		conform	conform
Purity test (TLC), SOP 211235		conform	conform
Water content, (micro determination, coulometric titration), Ph.Eur. 10.0., 2.5.32, SOP 304291 Vers. 2018-01: Mean value	%		5.1
Escin (HPLC), method 1 (AU %), SOP 400649	%	≥ 75.00	82.83

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Test	Unit	Specified value	Testresult
Inorganic impurities, (ICP-MS), for reference substances, SOP 811701: Calcium	%		<0.1
Potassium	%		<0.1
Magnesium	%		<0.1
Sodium	%		<0.1
Phosphorus	%		<0.1
Aluminium	%		<0.1
Residual solvents, (headspace-GC), SOP 805765:	%		
Residual solvents (LOQ: 0.050)			<0.050
Content, SOP 890000, calculated in (%): (100 - water - residual solvents - inorganic impurities) x chromatographic purity / 100	%		79
Content escin (HPLC-UV), expressed as protoescigenin, SOP 890013	%		53

This PhytoLab phyproof© reference standard is by definition a primary reference standard and does not need to be qualified against any other reference standard. The identity of the reference standard has been substantiated by at least two independent analytical methods such as IR, NMR, UV or MS analysis. A mass balance approach, which takes chromatographic purity into account, as well as the contents of water, residual solvents, inorganic impurities, and the counter ion (if the reference standard is present as a salt) is applied in the calculation of the absolute purity as given in this COA (see description of SOP 8900XX).

The absolute purity value (and not just the chromatographic purity result obtained by means of HPLC or GC) must be used in all quantitative calculations as the chromatographic

techniques do not yet account for water, residual solvents and inorganic impurities.

Escin is by definition a mixture of structurally related saponins. For the determination of absolute purity a variety of non-related assays can be applied, including a titration & loss on drying according to German Pharmacopoeia and a chromatographic purity by HPLC according to European Pharmacopoeia. Alternatively, the purity can also be expressed as protoescigenin. Depending on the assay method a different absolute purity of this reference substance has to be taken into account, as the assay methods are based on different non-related principles. The assay methods as well as the specifications in DAB, EP and USP are or were different. Therefore, please also pay close attention to the detailed information given on the accompanying data sheet and supplements to this COA. Please note that PhytoLab also offers a protoescigenin reference substance, our product # 83898. Specifications for horse chestnut and horse chestnut extract in the European Pharmacopoeia are related to protoescigenin. The use of a protoescigenin reference standard would thus also allow the determination of the content of escin, calculated as protoescigenin.

Vestenbergsgreuth, 30/Aug/2023

Katharina Kleiber

QC Reference Substances

This is a computer print and valid without signature. A signed certificate of analysis can be taken on request.

PhytoLab
SAFEGUARDING BOTANICAL QUALITY.

Certificate of analysis

Article:	89871 Escin
Material batch:	9758

Further information:

Shelf life/stability: The stated expiry date applies when the reference substance is stored in the original unopened container within the specified temperature range. PhytoLab does not guarantee the stability of the reference substance once the vial has been opened.

Long-term storage and handling: The reference standard should be stored in the original unopened vial, protected against light and humidity in an airtight container, within the temperature range given on the label and accompanying data sheet. If stored below room temperature, the vial should be warmed up to room temperature in a desiccator before it is opened in order to avoid condensation of humidity. The user assumes responsibility for deciding how previously opened reference standard vials should be used and the user must ensure that the contents of opened vials are still suitable for their intended use.

Exact weight: the exact weight of each vial is given on the label of the inner vial to two decimal places. This information may be used to produce stock solutions of a known concentration without having to weigh in the reference substance again. If used for this purpose, the content of the vial must be quantitatively transferred to a volumetric flask and filled up to the required level. Please note that PhytoLab is unable to guarantee the stability of the reference standard in solution.

Intended use: this reference standard is solely intended for laboratory analytical purposes, research & development, and scientific teaching and training purposes. It may not be used for any other purpose and particularly not for use in, or the production of, food, animal feed, human or veterinary drugs, cosmetics, medicinal products or diagnostic agents, including in-vitro diagnostic agents. PhytoLab is unable to guarantee the suitability of this reference standard for any particular application other than its qualitative and quantitative use in chromatography and identification testing.

Further information about this reference standard can be found on the accompanying data sheet or in our webshop. Spectral and chromatographic data, and a description of the applied chromatographic method, are provided in the attachments to this COA. A detailed explanation of all data given on the COA can be found in the guide that is available from the download area in our webshop, where you can also download all of the safety data sheets.

Product Data Sheet

Escin

Product #: 89871

Escin is a mixture of structurally related saponins

Physicochemical Data

CAS #:	6805-41-0
Molecular formula:	-
Molecular weight [g/mol]:	
Substance class:	Isoprenoids
Subgroup 1:	Terpenoid-type
Subgroup 2:	Triterpenes
Subgroup 3:	Triterpene saponins
Solubility:	soluble in ethanol; slightly soluble in water Please note that this solubility information is based on in-house experience or taken from published data. It is not meant to guarantee solubility up to a specific concentration, nor does it guarantee stability of the reference substance in solution.

Additional Information

Please note:	Escin reference substance is a mixture of structurally related saponins. A variety of assays are performed, including titration & loss on drying according to German Pharmacopoeia and a chromatographic purity by HPLC according to European Pharmacopoeia. The content expressed as protoescigenin is shown on the certificate of analysis as well. Please note that PhytoLab does also offer a protoescigenin reference substance, our product # 83898. Specifications for horse chestnut and horse chestnut extract in the European Pharmacopoeia are related to protoescigenin. The use of a protoescigenin reference standard would thus also allow the determination of the content of escin, calculated as protoescigenin.
Handling instructions:	Please note: For the quantitative analysis of escin a wide variety of non-related analytical methods are commonly being used, i.e. a titration, a photometric assay and an HPLC method. The content can be expressed as escin or as protoescigenin. Depending on the assay method a different absolute purity of this reference substance has to be taken into account, as the assay methods are based on different non-related principles. The assay methods as well as the specificatons in DAB, EP and USP are or were different. Detailed informations are, therefore, given on the accompanying documents.
Source:	botanical origin

Long-term storage conditions: 15-25 °C

Manufacturer:
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Germany

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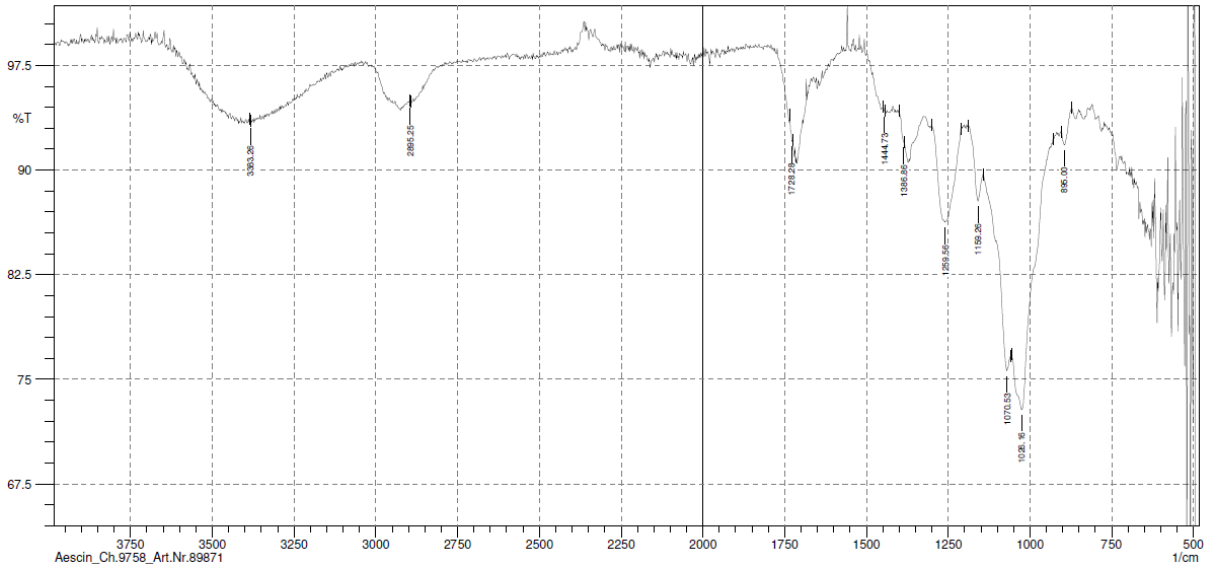
Mail: ref-substances@phytolab.de
Shop: https://phyproof.phytolab.com

Supplements

Escin **Batch # 9758**
Product # 89871

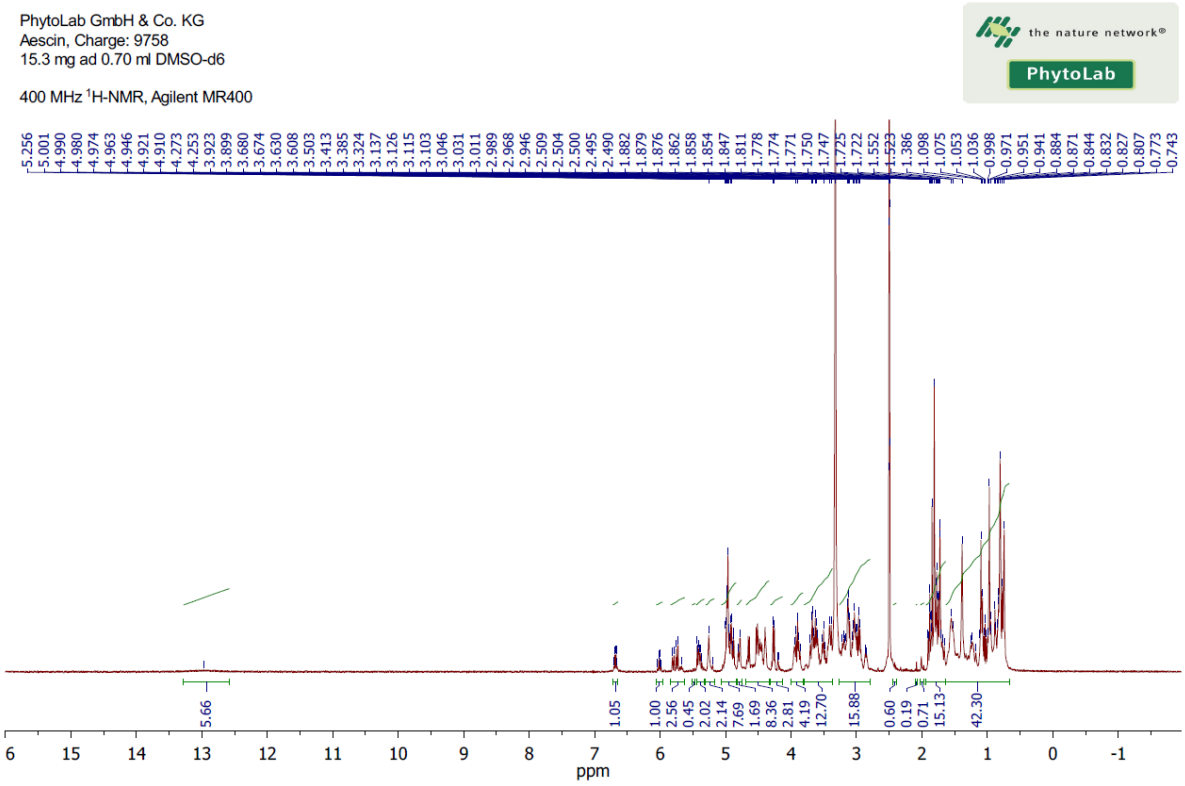
Identity tests:

IR spectrum

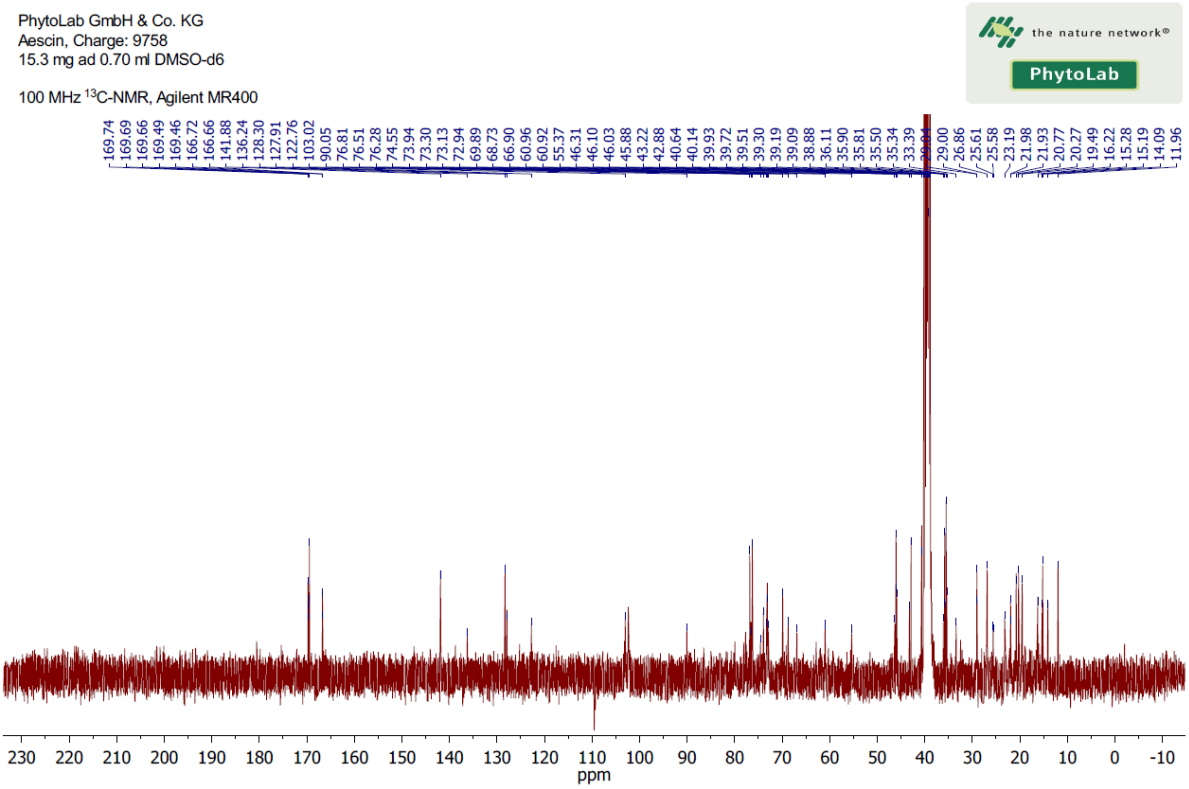


NMR spectra

^{1}H-NMR



^{13}C-NMR



Analytical background:

The original **photometric assay** for escin was developed in 1966. It is based on a color reaction between escin and a solution of ferric chloride in a mixture of anhydrous acetic acid and sulphuric acid. The color intensity that develops after the mixture has been heated for a specific time and at a specific temperature is determined photometrically. The chemical structure of the resulting red-coloured products is not completely clear. The reaction probably cleaves the sugar moieties and the resulting escin aglycones are expected to react further, producing double bond-containing reaction products while eliminating a water molecule. It is important to know that this reaction is based on the saponin structure of the escin. Furthermore, this reaction depends very much on the exact temperature and time, so reproducibility can be a challenge. Thus, in USP (and previously also in EP) this reaction is performed in parallel with a reference substance to produce a calibration curve, rather than performing this assay using the specific absorbance value for the reaction products.

An escin reference substance to be used in this photometric assay was required to fulfill the specifications layed out in the **German Pharmacopeia (DAB)**. The German Pharmacopoeia until today requires escin to have a content between 96.0 and 103.0 %, based on the anhydrous substance, the content being assigned by **titration**. This titration however is a titration of the glucuronic acid moieties of the escin components. Thus, it is not related to the saponin structure and thus also not related to the photometric assay.

For many years the **European Pharmacopoeia** aims to replace conventional, non-specific methods with more scientifically sound instrumental procedures. Thus, a method based on **HPLC analysis** of escin has been proposed for the first time in Pharmeuropa in 2008. In this method, all peaks eluting between two marker compounds, i.e. methyl salicylate and ibuprofen, are considered to be escin components. This is once again a convention and results in lower purity values assigned to escin reference substance, as well as in overall lower content values in horse chestnut, horse chestnut extracts and finished products prepared thereof. This HPLC assay again has no direct correlation neither to the photometric assay nor to the titration values.

In 2014, the respective horse chestnut monographs were updated, and both the photometric as well as the HPLC assay had to be performed in parallel for some period of time. Finally, with the publication of EP 9.0, the photometric assay was eliminated from the monographs completely, and as of today the HPLC assay is the only valid assay in the European Pharmacopoeia. The specifications for the content of escin, now calculated as protoescigenin, changed considerably. They were lowered from 3.0 % to 1.5 % in monograph 1830 (horse chestnut), and to 6.5 - 10.0 % from 16.0 - 20.0 % in monograph 1829 (horse-chestnut dry extract, standardised) as a result of the change of the analytical methodology.

Consequently, the European Pharmacopeia Escin CRS is now defined by a purity value calculated as protoescigenin. For our **phyproof® Reference Substance** Escin, product 89871, we have followed the same establishment procedure as European Pharmacopoeia, and on its certificate of analysis the purity as calculated in comparison to our primary reference substance protoescigenin, product 83898, is certified as well.

United States Pharmacopoeia, on the other hand, still applies the photometric assay as outlined above.

Depending on your usage of the escin reference substance, please use the following values in your assay:

HPLC assay according to the method published in European Pharmacopoeia:

Use the assigned absolute purity given on the COA under SOP 890000. This value is assigned considering the content of water, residual solvents and inorganic impurities as well as HPLC purity as determined by the method published in European Pharmacopoeia.

Photometric assay as described in United States Pharmacopoeia:

USP instructs to use the content value for escin on the anhydrous basis during the calculation. Thus, for the current batch of escin, batch # 9758, please use a value of 0.993 mg/mg of material = 99.3 %. This value is shown on the COA under SOP 402027.

Photometric assay as **previously** described in German Pharmacopoeia (DAB) or in the European Pharmacopeia monographs on Horse chestnut or Horse chestnut dry extract, standardised:

Use the substance as is, without correcting the purity value. The specification as required in DAB are fulfilled, the titration assay gives a value of 99.3 % for the anhydrous substance. The requirement is a content of 96 to 103 %, so the reference substance is well within these specifications, but in the assay the result of the titration is not used in the calculation of the results, the substance is simply considered to be 100 %. Please note that there is no current horse chestnut monographs in DAB. Still, such method may be described in your internal SOP.

Content calculated as protoescigenin according to the European Pharmacopoeia monographs:

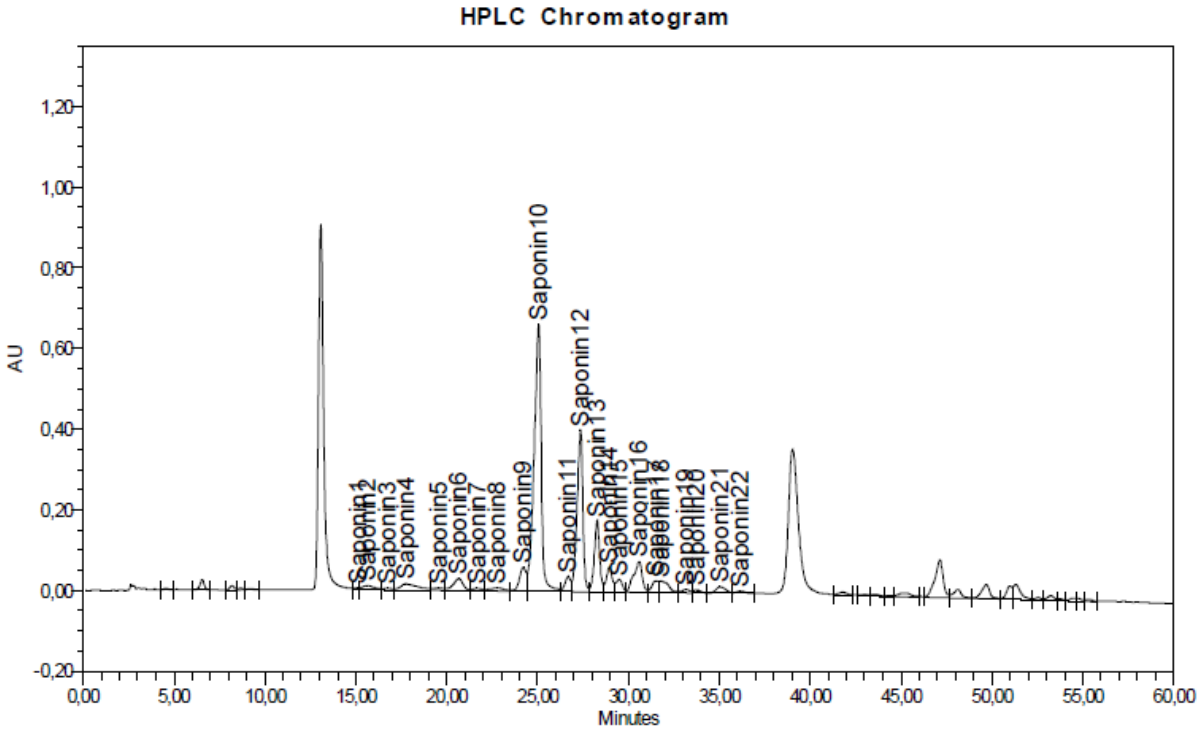
PhytoLab followed the same establishment procedure as European Pharmacopoeia using phyproof® Reference Substance protoescigenin, product # 83898, batch # 11222. The content of escin, calculated as protoescigenin, is shown on the COA under SOP 890013.

Please keep in mind that the results of the different assays are not be comparable. A specification of escin content in any product by the photometric assay will usually be higher than a specification by HPLC, and vice versa.

Please note that PhytoLab does also offer a **protoescigenin** reference substance, our product # 83898. Specifications for horse chestnut and horse chestnut extract in the European Pharmacopoeia are related to protoescigenin. The use of a protoescigenin reference substance would thus also allow the determination of the content of escin, calculated as protoescigenin.

If you have any doubt, please do not hesitate to contact us.

Chromatographic purity:



Peak Results

	Name	RT	Area	% Area
1		4,541	63345	0,12
2		6,542	403379	0,74
3		8,185	187537	0,35
4		8,661	85688	0,16
5		9,166	63172	0,12
6	Saponin1	15,143	103728	0,19
7	Saponin2	15,635	504063	0,93
8	Saponin3	16,741	127578	0,23
9	Saponin4	17,760	1083442	2,00
10	Saponin5	19,570	276816	0,51
11	Saponin6	20,663	1138389	2,10
12	Saponin7	21,672	221382	0,41
13	Saponin8	22,778	428811	0,79
14	Saponin9	24,211	1507336	2,78
15	Saponin10	25,064	18201538	33,52
16	Saponin11	26,684	817341	1,51
17	Saponin12	27,368	8958289	16,50
18	Saponin13	28,282	3707295	6,83
19	Saponin14	28,949	1389742	2,56
20	Saponin15	29,499	777644	1,43
21	Saponin16	30,588	2503436	4,61
22	Saponin17	31,504	690143	1,27
23	Saponin18	31,792	1058770	1,95
24	Saponin19	33,160	226131	0,42
25	Saponin20	33,756	186774	0,34
26	Saponin21	35,062	568157	1,05
27	Saponin22	36,158	182999	0,34
28		41,800	286152	0,53
29		43,033	112562	0,21
30		43,576	149988	0,28
31		44,469	72274	0,13
32		45,182	493637	0,91
33		47,137	3003266	5,53
34		48,126	711069	1,31
35		49,697	1151992	2,12
36		50,984	690685	1,27
37		51,317	1144180	2,11
38		52,537	156712	0,29
39		53,243	335599	0,62
40		53,772	93818	0,17
41		54,492	157796	0,29
42		54,702	142672	0,26
43		55,330	139649	0,26
Sum				100,00

Analytical conditions

Column:	Vydac RP18 , 250 x 4.6 mm, 5 µm
Mobile Phase:	eluent A: 0.05 % trifluoroacetic acid eluent B: CH_3CN
Mode:	gradient

Time [min]	Eluent A [%]	Eluent B [%]
0	70	30
15	70	30
25	65	35
35	65	35
65	50	50
70	10	90
75	10	90
78	70	30
85	70	30

Flow:	1.0 ml/min
Injection Volume:	20 µl
Column Temperature:	25 °C
Sample concentration:	approx. 509.7 mg/100 ml
Sample preparation:	dissolved in internal marker solution (IMS)
Detection:	UV, 210 nm
Special note:	Manufacturing solvent mixture: CH_3CN/ 0.05 % trifluoroacetic acid (40:60 V/V) Manufacturing IMS: dissolve 25.0 mg of methyl salicylate R and 75.0 mg of ibuprofen R in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 5.0 mL of the solution to 25.0 mL with the solvent mixture

Please note: Values on the certificate of analysis may vary as these are average values of at least six injections while above chromatogram and report is only one example. Non-integrated peaks originate from the blank injection.