

Anhang 3

Prüfbericht Sensibilisierungstest (LLNA) mit Produkt P-4G

BIOSERVICE

SCIENTIFIC
LABORATORIES
GmbH

Test for Sensitization
(Local Lymph Node Assay - LLNA)

with

Produkt P-4G

Report

BSL BIOSERVICE Project No.: 053226C

Sponsor

Bundesanstalt für Arbeitsschutz und Arbeitsmedizin (BAuA)

Friedrich-Henkel-Weg 1-25

44149 Dortmund

Germany

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The test results relate only to the items tested-

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Amtsgericht München, HRB 109 770

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ARBEITSMEDIZIN UND SICHERHEITSTECHNIK**

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GLP-Bescheinigung/Statement of GLP Compliance
(gemäß/according to § 19b Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 88/320/EG wurde durchgeführt in:

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 88/320/EEC at:

Prüfeinrichtung/Test facility Prüfstandort/Test site

BSL Bioservice Scientific Laboratories GmbH
Behringstrasse 6
82152 Planegg

(Unverwechselbare Bezeichnung und Adresse/Unequivocal name and address)

Prüfungen nach Kategorien/Areas of Expertise
(gemäß/according ChemVwV-GLP Nr. 5.3/OECD guidance)

- 2 Prüfungen auf toxische Eigenschaften
- 3 Prüfungen auf mutagene Eigenschaften (in vitro/in vivo)
- 9 Sonstige Prüfungen:
 - a) Mikrobiologische Sicherheitsprüfungen
 - b) Wirksamkeitsprüfungen an Zellkulturen

Datum der Inspektion/Date of Inspection

(Tag Monat.Jahr/day.month year)

11./12.02.2004

Die/Der genannte Prüfeinrichtung/Prüfstandort befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht

The above mentioned test facility/test site is included in the national GLP Compliance Programme and is inspected on a regular basis.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung/ diesem Prüfstandort die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können.

Based on the inspection report it can be confirmed, that this test facility/test site is able to conduct the aforementioned studies in compliance with the Principles of GLP.

München, 21.07.2004

IV.
Ritter
Leitender Gewerbedirektor



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Preface

General

Sponsor:	Bundesanstalt für Arbeitsschutz und Arbeitsmedizin (BAuA) Friedrich-Henkel-Weg 1-25 44149 Dortmund Germany
Study Monitor:	Mrs Heidi Ott
Test Facility:	BSL BIOSERVICE Scientific Laboratories GmbH Behringstraße 6 82152 Planegg Germany
BSL BIOSERVICE- Project No.:	053226C
Test Item:	Produkt P-4G
Title:	Test for Sensitization (Local Lymph Node Assay - LLNA) with Produkt P-4G

Project Staff

Study Director:	Dr. Ingrid Haist
Deputy Study Directors:	Dr. Daniela Brummer Dr. Achim Albrecht
Management:	Dr. Wolfram Riedel Dr. Angela Lutterbach
Quality Assurance Unit:	Dipl. Biol. Uwe Hamann Dr. Margarete Hoechst Dr. Helga Köhn

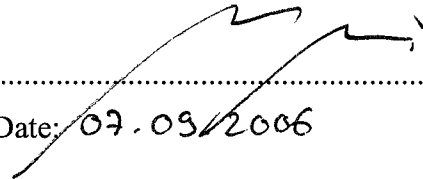
Schedule

Arrival of Test Item:	December 29, 2005
Date of Draft Project Protocol:	December 27, 2005
Date of Project Protocol:	January 23, 2006
Start of Study (preliminary test):	February 08, 2006
End of Study:	March 08, 2006
Date of Draft Report:	May 09, 2006
Date of Report:	September 07, 2006

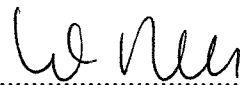
Project Staff Signatures

Study Director:

Dr. Ingrid Haist


.....
Date: 07.09.2006

Management:


.....
Date: Sept. 8, 2006

Quality Assurance

GLP Compliance

This study was conducted to comply with:

Chemikaliengesetz (“Chemicals Act”) of the Federal Republic of Germany, Appendix 1 to § 19a as amended on May 08, 2001. Published May 14, 2001 in Bundesgesetzblatt 2001 part I no. 21, pp. 844 – 854.

OECD Principles of Good Laboratory Practice (as revised in 1997); OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1.

Environment Directorate, Organisation for Economic Co-operation and Development, Paris 1998.

This study was assessed for compliance with the project protocol, the study plan and the Standard Operating Procedures of BSL BIOSERVICE. The study and/or the test facility were periodically inspected by the Quality Assurance Unit and the dates and phases of the inspections and audits are included in this report. These inspections and audits were carried out by the Quality Assurance Unit, personnel independent of staff involved in the study. The final report of the study was audited. A Quality Assurance Statement, signed by the Quality Assurance, is included in this report.

Guidelines

This study followed the procedures indicated by the following internationally accepted guidelines and recommendations:

OECD Guidelines for Testing of Chemicals, number 429 “Skin Sensitization: Local Lymph Node Assay” (adopted: 24th April 2002).

EPA Health Effects Test Guidelines, OPPTS 870.2600 “Skin Sensitization“, EPA 712-C-03-197, March 2003.

Archiving

The following records will be stored in the scientific archives of BSL BIOSERVICE Scientific Laboratories GmbH according to the GLP-Regulations:

A copy of the final report, the project protocol, the study plan and a documentation of all raw data generated during the conduct of the study (documentation forms as well as any other notes of raw data, printouts of instruments and computers) and the correspondence with the sponsor concerning the project. Default archiving period for the study documentation is 15 years.

If test item is left over a sample will be stored according to the GLP-Regulations. Samples that are unstable may be disposed of before that time. No raw data or material relating to the study will be discarded without the sponsor's prior consent. Remaining test item will be returned to the sponsor as requested.

Statement of Compliance

BSL BIOSERVICE-
Project No.: 053226C

Test Item: Produkt P-4G

Title: Test for Sensitization
(Local Lymph Node Assay - LLNA) with
Produkt P-4G

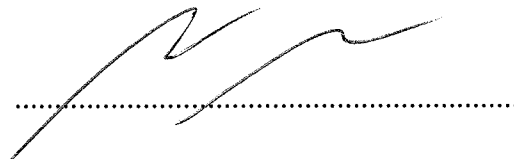
Study Director: Dr. Ingrid Haist

This study performed in the test facility BSL BIOSERVICE Scientific Laboratories GmbH was conducted in compliance with Good Laboratory Practice Regulations:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to § 19a as amended on May 08, 2001. Published May 14, 2001.
"OECD Principles of Good Laboratory Practice (as revised in 1997)", Paris 1998.

There were no circumstances that may have affected the quality or integrity of the study.

Study Director: Dr. Ingrid Haist



Date: 04.10.2006

This statement does not include the preliminary test.

Statement of the Quality Assurance Unit

BSL BIOSERVICE-
Project No.: 053226C

Test Item: Produkt P-4G

Title: Test for Sensitization
(Local Lymph Node Assay - LLNA) with
Produkt P-4G

Study Director: Dr. Ingrid Haist

This report was audited by the Quality Assurance Unit and the conduct of this study was inspected on the following dates:

<i>Phases of QAU Inspections</i>	<i>Dates of QAU Inspections</i>	<i>Dates of Reports to the Study Director and Management</i>
Audit Project Protocol/ Study Plan:	January 26, 2006	January 26, 2006
Experimental Phase Audit (Method Audit):	January 19, 2006	January 19, 2006
Draft Report Audit:	May 15, 2006	May 15, 2006
Report Audit:	September 27, 2006	September 27, 2006

This report reflects the raw data.

Member of the
Quality Assurance Unit:

..... Helga Köhn

Date: Sept 27, 2006

This statement does not include the preliminary test.

Summary

Four concentrations were chosen to gain a wide spectrum for the test design:

Due to results of a solubility test and in consultation with the sponsor, the test item was assayed at concentrations of 15%, 9%, 3% and 1% (w/v). A positive control group was carried along in accordance to demonstrate appropriate performance of the assay.

The vehicle was AOO (3+1 (v/v) Acetone/Olive Oil). Stability of the test item in the vehicle was proven (details see Annex to this report).

Each mouse was treated by topical application of the prepared test item to the entire dorsal surface of each ear once daily over three consecutive days.

Five days after the first topical application all mice were injected intravenously with ³H-methyl thymidine.

Directly prior to the first application and shortly before excising the lymph nodes the thickness of both ears from all animals was measured.

No different ear thickness development between test and control groups could be found.

Approximately 5 hours after ³H-methyl thymidine-injection all mice were sacrificed and the draining “auricular lymph nodes” were excised and weighed individually.

No different lymph node weights between test and control groups could be found.

A single cell suspension of the lymph node cells for each animal was prepared. The ³H-methyl thymidine – incorporation was measured in a β-counter and expressed as the number of disintegrations per minute (DPM). Determination of radioactivity was performed individually for each animal.

The proliferative response of lymph node cells was calculated as the ratio of ³H-methyl thymidine - incorporation into lymph node cells of test group animals relative to that recorded for control group animals. A stimulation index, ratio of test item / negative control, was calculated for each concentration.

None of the tested concentrations of the test item reached the stimulation index of 3.

The stimulation index at a concentration	of	15%	was	2.5
The stimulation index at a concentration	of	9%	was	1.9
The stimulation index at a concentration	of	3%	was	2.5
The stimulation index at a concentration	of	1%	was	2.4
The mean stimulation index of the 4 concentrations 1%, 3%, 9% and 15% was				2.3

All animals showed the expected weight development, which includes a weight loss of up to 2 g throughout the study.

At the daily clinical observation the animals did not show any visible clinical symptoms.

Conclusions

The EC3 value could not be calculated as the stimulation indices of all concentrations were below 3. This finding was confirmed by the second endpoint, the weight of the lymph nodes, as none of the test groups showed increased lymph node weights compared to the control group.

Consequently, according to OECD 429 the test item Produkt P-4G has shown no skin sensitizing properties under the given experimental conditions.

Introduction

The LLNA has been developed as an alternative method for the identification of skin sensitizing test items and measures the proliferation of lymphocytes isolated from lymph nodes (auricular lymph nodes) draining the site of exposure (dorsal aspect of the ears) in mice.

Lymphocyte proliferation is measured by determining the incorporation of ³H-methyl thymidine.

This experiment being on hand was conducted in order to receive a quantitative result of the sensitizing properties of the test item. This is necessary for the Federal Institute of Occupational Safety and Health (Bundesanstalt für Arbeitsschutz und Arbeitsmedizin, BAuA) to evaluate a ranking system for dyestuffs which have shown positive results in the Guinea Pig Maximisation Test (GPMT) of Magnusson and Kligman.

No validated *in vitro* method is available for assessing sensitization potency.

Materials and Methods

Characterisation of the Test Item

The test item and the information concerning the test item were provided by the sponsor.

Substance name:	Produkt P-4G
Product:	Produkt P-4G
Notification number:	00 04 1291
CAS-No.:	185461-17-0
Batch No.:	BASF 426/6
Chemical name:	Lithium sodium 4-(5-(4-chloro-6-(3sulfonatophenylamino)-1,3,5-triazine-2ylamino)-2-sulfonatophenylazo)-5-oxo-1-(4-sulfonatophenyl)-4,5-dihydro-1H-pyrazole-3-carboxylate
Active components:	48.5%
Colour:	yellow
Physical state:	powder
Purity:	48.5%
Unidentified components:	not specified
Stability:	March 2005 (expiration date of the certificate; specified on the package), therefore stability of the test item was additionally proofed (see Annex II to this study)
Storage:	at room temperature
Safety Precautions:	Routine hygienic procedures were sufficient to assure personnel health and safety

Preparation of the Vehicle

The vehicle was AOO (3+1 (v/v) Acetone/Olive Oil).

(Acetone; CAS 67-64-1, Merck, Lot K32732014; Olive Oil Highly refined, Sigma GmbH, Lot 115K6046)

Preparation of the Test Item

The test item was assayed at four concentrations.

A solubility test was performed first to detect the highest possible application solution.

Due to results of the solubility test and in consultation with the sponsor, the applied concentrations of the homogenous suspension were:

15%, 9%, 3% and 1% (w/v).

The preparations were made immediately prior to each dosing. The two components of the vehicle were confected first. Afterwards the test item was weighed out and the appropriate amount of vehicle was added.

Stability of the Test Item in the Vehicle

Analysis of Stability was performed at the contract laboratory BioProof AG, Weihenstephanerstr. 28, 81673 München, Germany.

Controls

The vehicle served as negative control.

P-Phenylenediamine (CAS 106-50-3, Sigma GmbH, purity > 98%) at a concentration of 1% (w/v) in AOO (3+1 (v/v) Acetone/Olive Oil) served as positive control.

Other Materials

³H-methyl thymidine (TRK 300, 25 Ci/mmol; Lot B264; Amersham Pharmacia Biotech), diluted to a working concentration of 80µCi/mL

NaCl 0.9%, B. Braun Melsungen AG, Lot 5361A191

Trichloroacetic acid (TCA), BSL BIOSERVICE Lot 240106

Phosphate buffered saline (PBS), BSL BIOSERVICE Lot 080206

Test Animals

Mice, CBA/Ca01aHsd, female, age 6 – 12 weeks, 5 mice per test group.

The animals were derived from a controlled full barrier maintained breeding system (SPF).

Source: Harlan Winkelmann GmbH, D-33178 Borcheln.

According to Art. 9.2, No.7 of the German Act on Animal Welfare the animals are bred for experimental purposes.

Animal Husbandry

The animals were barrier maintained (semi-barrier) in an air conditioned room

- Temperature: 22 ± 3 °C
- Rel. humidity: $55 \pm 10\%$
- Artificial light, sequence being 12 hours light, 12 hours dark
- Air change: at least 10 x / hour
- Feeding ad libitum, ssniff R/M-H, 10 mm V1534-000 complete diet for rats/mice, totally-pathogen-free (TPF)
- Free access to tap water (drinking water, municipal residue control, microbiol. controlled periodically)
- The animals were kept in groups in Macrolon-cages on Lignocel bedding
- Certificates of food, water and bedding are filed at BSL Bioservice
- Adequate acclimatisation period

Preparation of the Animals

The animals were randomly selected.

Identification was ensured by cage number and individual marking (tail).

Clinical Observation

Prior to the application and once a day thereafter all animals were observed in order to detect special clinical signs or reactions to treatment.

Weight Assessment

The animals were weighed prior to the application and at the end of the test period.

Dose Groups

4 Test Groups (4 different concentrations), 1 Positive Control Group and 1 Negative Control Group (vehicle) were tested.

Test Regime

Topical Application

Before the first application the thickness of the ears of all animals were determined by using a digital caliper gage. Then each mouse was treated by topical application of 25 μ L of the selected solution to the entire dorsal surface of each ear.

Topical applications were performed once daily over three consecutive days.

Administration of ^3H -methyl thymidine

Five days after the first topical application treatment all mice were dosed with 20 μ Ci ^3H -methyl thymidine by intravenous injection (tail vein) of 250 μ L of ^3H -methyl thymidine, diluted to a working concentration of 80 μ Ci/mL.

Preparation of cell suspension

Approximately 5 hours after ^3H -methyl thymidine-injection all mice were sacrificed. Before excising the lymph nodes the thickness of the ears of all animals was determined. The draining “auricular lymph nodes” were excised, pooled for each animal (2 lymph nodes per animal, if technically possible), weighed and collected in PBS. A single cell suspension of pooled lymph node cells was prepared by gentle mechanical disaggregation through polyamide gauze (200 mesh size). After washing the gauze with PBS the cell suspension was pelleted in a centrifuge. The supernatant was discarded and the pellets were resuspended with PBS. This washing procedure was repeated.

After the final wash each pellet was resuspended in approx. 1 mL 5% TCA at approx. 4 °C overnight for precipitation of macromolecules. Each precipitate was once washed again, resuspended in 10 mL scintillation fluid, transferred into scintillation vials and stored at room temperature overnight.

Determination of incorporated ^3H -methyl thymidine

The ^3H -methyl thymidine – incorporation was measured in a β -counter and expressed as the number of disintegrations per minute (DPM). Similarly, background ^3H -methyl thymidine levels were also measured (5% TCA). Determination of radioactivity was performed individually for each animal.

Evaluation of Results

The proliferative response of lymph node cells was expressed as the number of radioactive disintegrations per minute per lymph node (DPM/NODE) and as the ratio of ³H-methyl thymidine - incorporation into lymph node cells of test group animals relative to that recorded for control group animals (STIMULATION INDEX). Before DPM/NODE values were determined, background values were subtracted.

EC3 values, calculated concentrations which induce stimulation indices of three, are determined by linear interpolation $\{EC3 = c + [(3-d) / (b-d)] \times (a-c)\}$, between two points of the stimulation indices axis, one above (a,b) and one below (c,d) the stimulation index of three. If all measured points are above or below the stimulation index of three, no EC3 value can be stated.

A substance is regarded as a 'sensitizer' in the LLNA if at least one concentration of the test item results in a 3 fold or greater increase in ³H-methyl thymidine - incorporation into lymph node cells of the lymph nodes of the test group animals, relative to that recorded for the lymph nodes of control group animals (**Stimulation Index equal to or greater than 3.0**).

Deviation to the Project Protocol

There was no deviation to the project protocol.

Results

Four concentrations were chosen to gain a wide spectrum for the test design:

Due to results of a solubility test and in consultation with the sponsor, the test item was assayed at concentrations of 15%, 9%, 3% and 1% (w/v). Additionally a positive control for verifying the functionality of the current test run was carried along.

The vehicle was AOO (3+1 (v/v) Acetone/Olive Oil). Stability of the test item in the vehicle was proven (details see Annex to this report).

Each mouse was treated by topical application of the prepared test item to the entire dorsal surface of each ear once daily over three consecutive days.

Five days after the first topical application all mice were injected intravenously with ³H-methyl thymidine.

Directly prior to the first application and shortly before excising the lymph nodes the thickness of both ears from all animals was measured. This is to exclude irritating properties of the test item, which may lead to false positive results.

Mean Ear thickness at:	day 1	day 6
of the 15% group was	0.20 mm	0.20 mm
of the 9% group was	0.21 mm	0.21 mm
of the 3% group was	0.20 mm	0.20 mm
of the 1% group was	0.21 mm	0.22 mm
of the negative control group was	0.20 mm	0.20 mm
of the positive control group was	0.19 mm	0.20 mm

Approximately 5 hours after ³H-methyl thymidine-injection all mice were sacrificed and the draining “auricular lymph nodes” were excised and weighed individually.

The mean weights of the lymph nodes

for the 15% group was	3.0 mg
for the 9% group was	2.7 mg
for the 3% group was	3.1 mg
for the 1% group was	3.3 mg
for the negative control-group was	2.6 mg
for the positive control-group was	5.5 mg

A single cell suspension of the lymph node cells for each animal was prepared. The ³H-methyl thymidine – incorporation was measured in a β-counter and expressed as the number of disintegrations per minute (DPM). Determination of radioactivity was performed individually for each animal.

The proliferative response of lymph node cells was calculated as the ratio of ³H-methyl thymidine - incorporation into lymph node cells of test group animals relative to that recorded for control group animals. A stimulation index, ratio of test item / negative control, was calculated for each concentration.

The stimulation index at a concentration of 15% was **2.5**

The stimulation index at a concentration of 9% was **1.9**

The stimulation index at a concentration of 3% was **2.5**

The stimulation index at a concentration of 1% was **2.4**

The stimulation of the positive control (Phenylenediamine) at a concentration of 1% was **9.9**

All animals showed the expected weight development, which includes a weight loss of up to 2 g throughout the study.

At the daily clinical observation the animals did not show any visible clinical symptoms.

Conclusions

The EC3 value (derived by linear interpolation) could not be calculated as the stimulation indices of all concentrations were below 3. This finding was confirmed by the second endpoint, the weight of the lymph nodes, as none of the test groups showed increased lymph node weights compared to the control group.

Consequently, according to OECD 429 the test item Produkt P-4G has shown no skin sensitizing properties under the given experimental conditions.

Table 1: Weight Gain (g)

<i>Group</i>	<i>Animal No.</i>	<i>Start of study</i>	<i>End of study</i>	<i>Weight gain</i>
<i>Produkt P-4G 15% in AOO</i>	1	18	19	1
	2	17	18	1
	3	16	17	1
	4	16	16	0
	5	15	16	1
<i>Produkt P-4G 9% in AOO</i>	6	16	16	0
	7	17	17	0
	8	18	18	0
	9	16	16	0
	10	16	16	0
<i>Produkt P-4G 3% in AOO</i>	11	17	17	0
	12	17	18	1
	13	16	16	0
	14	17	17	0
	15	17	18	1
<i>Produkt P-4G 1% in AOO</i>	16	18	19	1
	17	18	18	0
	18	16	17	1
	19	15	16	1
	20	16	16	0
<i>Negative control AOO</i>	26	17	18	1
	27	17	17	0
	28	16	16	0
	29	16	16	0
	30	17	18	1
<i>Positive control 1% in AOO</i>	21	17	17	0
	22	17	18	1
	23	16	17	1
	24	17	18	1
	25	18	19	1

Table 2a: Radioactive determination of the test substance groups.
If not noted individually, results include both lymph nodes of an animal.

POS	CPMA	LUM	CPM	Test Item	Conc. [%]	Animal number	DPM	DPM-mean background	DPM/Node	Stimulation Index
30	383.0	4	367.7	Negative Control		26	99.8	97.8	48.9	
31	282.0	6	265.1		27	72.0	69.9	35.0		
32	322.0	6	302.7		28	82.2	80.2	40.1		
33	230.0	9	209.3		29	56.8	54.8	27.4		
34	193.0	11	171.8		30	46.6	44.6	22.3		
MV	282.0		263.3		MV	71.5	69.5	34.7	1.0	
SD	67.0		68.9		SD	18.7	18.7	9.4		
61	750.0	3	727.5	Produkt P-4G	15	1	197.5	195.5	97.7	2.8
62	523.0	4	502.1			2	136.3	134.3	67.1	1.9
63	715.0	4	686.4			3	186.3	184.3	92.2	2.7
64	887.0	2	869.3			4	236.0	233.9	117.0	3.4
65	499.0	5	474.1			5	128.7	126.7	63.3	1.8
MV	674.8		651.9			MV	176.9	174.9	87.5	2.5
SD	145.8		147.1			SD	39.9	39.9	20.0	0.6
66	559.0	5	531.1	Produkt P-4G	9	6	144.2	142.1	71.1	2.0
67	514.0	3	498.6			7	135.3	133.3	66.7	1.9
68	752.0	3	729.4			8	198.0	196.0	98.0	2.8
69	448.0	4	430.1			9	116.7	114.7	57.4	1.7
70	359.0	6	337.5			10	91.6	89.6	44.8	1.3
MV	526.4		505.3			MV	137.2	135.2	67.6	1.9
SD	131.4		130.2			SD	35.3	35.3	17.7	0.5
73	552.0	5	524.4	Produkt P-4G	3	11	142.3	140.3	70.2	2.0
74	708.0	5	672.6			12	182.6	180.6	90.3	2.6
75	1106.0	2	1083.9			13	294.2	292.2	146.1	4.2
76	379.0	6	356.3			14	96.7	94.7	47.3	1.4
77	644.0	4	618.2			15	167.8	165.8	82.9	2.4
MV	677.8		651.1			MV	176.7	174.7	87.4	2.5
SD	241.0		241.6			SD	65.6	65.6	32.8	0.9
78	375.0	7	348.8	Produkt P-4G	1	16	94.7	92.7	46.3	1.3
79	393.0	6	369.4			17	100.3	98.3	49.1	1.4
80	1056.0	2	1034.9			18	280.9	278.9	139.5	4.0
81	903.0	2	884.9			19	240.2	238.2	119.1	3.4
82	506.0	5	480.7			20	130.5	128.5	64.2	1.8
MV	646.6		623.7			MV	169.3	169.3	84.7	2.4
SD	279.7		282.1			SD	76.6	76.6	38.3	1.1
150	22.0	50	11.0	Background Szinti and TCA			3.0			
151	14.0	100	0.0				0.0			
152	28.0	39	17.1				4.6			
153	20.0	65	7.0				1.9			
154	15.0	87	2.0				0.5			
MV			7.4		MV	2.0	0.0	0.0	0.0	0.0
SD			6.2		SD	1.7				

Szinti = scintillation fluid; TCA = trichloroacetic acid; MV = Mean Value, SD = Standard Deviation; DPM = disintegrations per minute, CPM = counts per minute chemiluminescence adjusted; CPMA = counts per minute including chemiluminescence; LUM = % chemiluminescence

Table 2b: Radioactive determination of the positive control group of the resent study. If not noted individually, results include both lymph nodes of an animal.

POS	CPMA	LUM	CPM	Test Item	Conc. [%]	Animal number	DPM	DPM-mean back-ground	DPM/Node	Stimulation Index
30	383.0	4	367.7	Negative Control		26	99.8	97.8	48.9	
31	282.0	6	265.1		27	72.0	69.9	35.0		
32	322.0	6	302.7		28	82.2	80.2	40.1		
33	230.0	9	209.3		29	56.8	54.8	27.4		
34	193.0	11	171.8		30	46.6	44.6	22.3		
MV	282.0		263.3		MV	71.5	69.5	34.7	1.0	
SD	67.0		68.9	SD	18.7	18.7	9.4			
25	2179.0	1	2157.2	P-Phenylene-diamine	1	1	585.6	583.6	291.8	8.4
26	3639.0	1	3602.6		2	2	977.9	975.9	488.0	14.0
27	2534.0	1	2508.7		3	3	681.0	679.0	339.5	9.8
28	2467.0	1	2442.3		4	4	663.0	660.9	330.5	9.5
29	2074.0	1	2053.3		5	5	557.3	555.3	277.7	8.0
MV	2578.6		2552.8		MV	692.9	690.9	345.5	9.9	
SD	557.3		551.8	SD	149.8	149.8	74.9	2.2		
150	22.0	50	11.0	Background Szinti and TCA			3.0			
151	14.0	100	0.0				0.0			
152	28.0	39	17.1				4.6			
153	20.0	65	7.0				1.9			
154	15.0	87	2.0				0.5			
MV			7.4		MV	2.0	0.0	0.0	0.0	
SD			6.2	SD	1.7					

Szinti = scintillation fluid; TCA = trichloroacetic acid; MV = Mean Value, SD = Standard Deviation; DPM = disintegrations per minute, CPM = counts per minute chemiluminescence adjusted; CPMA= counts per minute including chemiluminescence; LUM = % chemiluminescence

Table 2c: Radioactive determination of the latest BSL BIOSERVICE positive control

Study number 060114-3

P-Phenylenediamine (CAS 106-50-3, Sigma GmbH, purity > 98%); Lot 69H3638)

1% (w/v) in AOO (3+1 (v/v) Acetone/Olive Oil)

Date of Certificate: April 27, 2006

If not noted individually, results include both lymph nodes of an animal.

POS	CPMA	LUM	CPM	Test Item	Conc. [%]	Animal number	DPM	DPM-mean back-ground	DPM/Node	Stimulation Index
18	366.0	4	351.4	Negative Control		16	95.4	94.3	47.2	
19	323.0	4	310.1			17	84.2	83.1	41.6	
20	666.0	2	652.7			18	177.2	176.1	88.1	
21	180.0	7	167.4			19	45.4	44.4	22.2	
22	320.0	4	307.2			20	83.4	82.3	41.2	
MV	371.0		357.7			MV	97.1	96.1	48.0	1.0
SD	160.3		160.1		SD	43.4	43.4	21.7		
78	3210.0	0	3210.0	P-Phenylenediamine	1	1	871.3	870.3	435.1	9.1
79	2417.0	1	2392.8			2	649.5	648.5	324.2	6.8
80	2043.0	1	2022.6			3	549.0	548.0	274.0	5.7
81	1633.0	1	1616.7			4	438.8	437.8	218.9	4.6
82	3544.0	0	3544.0			5	962.0	961.0	480.5	10.0
MV	2569.4		2557.2			MV	694.1	693.1	346.6	7.2
SD	712.4		720.7	SD	195.6	195.6	97.8	2.0		
85	29.0	48	15.1	Background Szinti and TCA			4.1			
86	15.0	80	3.0				0.8			
87	11.0	100	0.0				0.0			
88	15.0	100	0.0				0.0			
89	18.0	94	1.1				0.3			
MV			3.8			MV	1.0	0.0	0.0	0.0
SD			5.7		SD	1.6				

Szinti = scintillation fluid; TCA = trichloroacetic acid; MV = Mean Value, SD = Standard Deviation; DPM = disintegrations per minute, CPM = counts per minute chemiluminescence adjusted; CPMA= counts per minute including chemiluminescence; LUM = % chemiluminescence

Table 3: Individual weight of each lymph node and means of animals and groups

<i>Group</i>	<i>Animal No.</i>	<i>right lymph node(mg)</i>	<i>left lymph node (mg)</i>	<i>Mean of individual animal (mg)</i>	<i>Mean of test group (mg)</i>
<i>Produkt P-4G 15% in AOO</i>	1	3.2	3.9	3.6	3.0
	2	2.3	3.0	2.7	
	3	2.9	2.9	2.9	
	4	3.9	3.2	3.6	
	5	2.7	2.1	2.4	
Mean value		3.0	3.0	3.0	
Standard Deviation		0.5	0.6	0.5	
<i>Produkt P-4G 9% in AOO</i>	6	2.8	3.8	3.3	2.7
	7	2.0	2.6	2.3	
	8	3.1	2.8	3.0	
	9	2.4	2.7	2.6	
	10	2.5	2.2	2.4	
Mean value		2.6	2.8	2.7	
Standard Deviation		0.4	0.5	0.4	
<i>Produkt P-4G 3% in AOO</i>	11	3.3	2.6	3.0	3.1
	12	3.7	3.1	3.4	
	13	2.5	4.3	3.4	
	14	2.4	2.6	2.5	
	15	3.0	3.3	3.2	
Mean value		3.0	3.2	3.1	
Standard Deviation		0.5	0.6	0.3	
<i>Produkt P-4G 1% in AOO</i>	16	3.2	3.3	3.3	3.3
	17	3.4	2.7	3.1	
	18	3.0	3.5	3.3	
	19	4.0	3.1	3.6	
	20	3.5	3.3	3.4	
Mean value		3.4	3.2	3.3	
Standard Deviation		0.3	0.3	0.2	
<i>Negative control AOO</i>	26	2.8	3.5	3.2	2.6
	27	2.7	2.6	2.7	
	28	2.7	2.6	2.7	
	29	2.2	2.2	2.2	
	30	2.1	2.5	2.3	
Mean value		2.5	2.7	2.6	
Standard Deviation		0.3	0.4	0.3	
<i>Positive control 1% in AOO</i>	21	4.2	5.3	4.8	5.5
	22	5.1	7.7	6.4	
	23	4.6	6.2	5.4	
	24	6.1	5.7	5.9	
	25	4.6	5.2	4.9	
Mean value		4.9	6.0	5.5	
Standard Deviation		0.7	0.9	0.6	

Table 4:
Individual measurement of ear thickness and means of animals and groups

Group	Animal No.	Measurement of ear thickness				Mean of first test group	Mean of second test group
		right		left			
		first	second	first	second		
<i>Produkt P-4G 15% in AOO</i>	1	0.21	0.21	0.20	0.20	0.20	0.20
	2	0.20	0.20	0.20	0.20		
	3	0.19	0.20	0.19	0.19		
	4	0.20	0.20	0.21	0.21		
	5	0.20	0.20	0.22	0.22		
<i>Produkt P-4G 9% in AOO</i>	6	0.22	0.22	0.22	0.23	0.21	0.21
	7	0.22	0.22	0.21	0.21		
	8	0.17	0.18	0.17	0.18		
	9	0.23	0.23	0.22	0.22		
	10	0.20	0.21	0.21	0.21		
<i>Produkt P-4G 3% in AOO</i>	11	0.17	0.17	0.17	0.18	0.20	0.20
	12	0.20	0.21	0.18	0.19		
	13	0.21	0.21	0.22	0.22		
	14	0.19	0.20	0.18	0.19		
	15	0.23	0.23	0.21	0.21		
<i>Produkt P-4G 1% in AOO</i>	16	0.23	0.23	0.21	0.21	0.21	0.22
	17	0.20	0.21	0.22	0.23		
	18	0.21	0.21	0.20	0.22		
	19	0.22	0.23	0.21	0.22		
	20	0.20	0.20	0.19	0.20		
<i>Negative control AOO</i>	26	0.20	0.21	0.19	0.20	0.20	0.20
	27	0.23	0.23	0.21	0.21		
	28	0.18	0.19	0.19	0.20		
	29	0.19	0.19	0.20	0.20		
	30	0.17	0.18	0.19	0.19		
<i>Positive control 1% in AOO</i>	21	0.22	0.22	0.20	0.21	0.19	0.20
	22	0.18	0.19	0.18	0.18		
	23	0.17	0.18	0.17	0.18		
	24	0.22	0.22	0.20	0.20		
	25	0.19	0.20	0.20	0.21		

Distribution of the Report

Sponsor 1x (original)

Study Director 1x (copy)

Annex

Annex I: Analytical Report: 19 pages

Annex II: HPLC-Reports: 5 pages and 2 pages

Prüfbericht nach DIN EN ISO/IEC 17025

Nr.: 11045

Auftraggeber:

DyStar Textilfarben GmbH & Co Deutschland KG
Ökologie, PS
Frau Dr. Erika Kunz
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Technische
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Akkreditiertes Prüflabor Analytik



Prüfgegenstand:

Kennzeichnung des Auftraggebers: RE 301
Probenbezeichnung: Produkt P4G/HPLC-MS

Auftragsnummer des Prüflabors: 11045
Prüfmerkmal: Molekülspektroskopie
Eingangsdatum: 2002-06-18
Prüfdatum: 2002-07-29
Prüfverfahren:

Ergebnis:

Siehe Anlage

Datum: 2002-07-29


(Auftragskoordination)

Die DACH Deutsche Akkreditierungsstelle Chemie GmbH hat dem oben genannten Prüflabor am 15.09.2000 bestätigt, dass es die Kompetenz nach DIN EN ISO/IEC 17025 besitzt. Prüfungen im Bereich chemische und chemisch-physikalische Analytik, anwendungstechnische und sicherheitstechnische Prüfungen durchzuführen. Das auf den Prüfgegenstand angewandte Prüfverfahren ist:

Bestandteil der Akkreditierung	A-Typ 1
<input checked="" type="checkbox"/> Bestandteil einer akkreditierten Prüfmethode	A-Typ 2
nicht Bestandteil der Akkreditierung	A-Typ 3

Die in diesem Prüfbericht genannten Ergebnisse beziehen sich ausschließlich auf die bezeichneten Prüfgegenstände. Die angegebenen Messunsicherheiten beziehen sich auf den Anlieferungszustand des Prüfgegenstands. Die Vervielfältigung (auch auszugsweise) dieses Prüfberichts bedarf der Zustimmung des Prüflabors.

**Prüfbericht nach DIN EN ISO/IEC 17025
(Anlage)**



Nr.: 11045

Ergebnis:

Produkt P4G Rückstellmuster

Bestimmung von Verunreinigungen analog mitgelieferter Analysenunterlagen
mittels HPLC -MS

Versuch der Identifizierung der einzelnen Komponenten.

Anlagen:

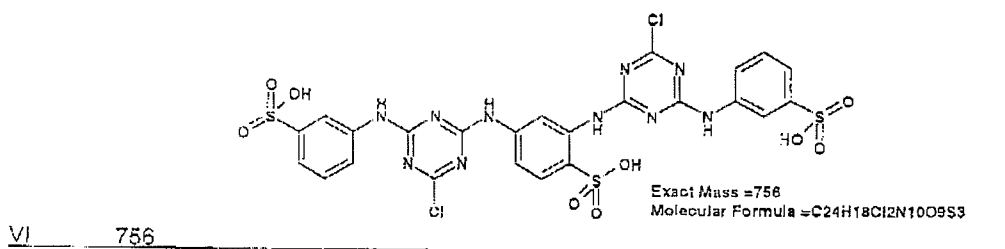
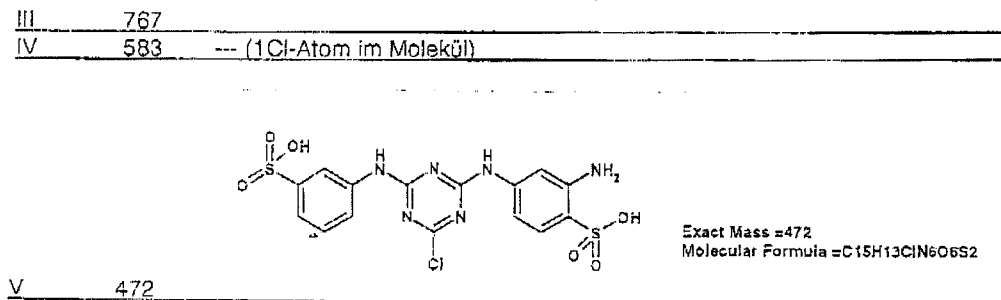
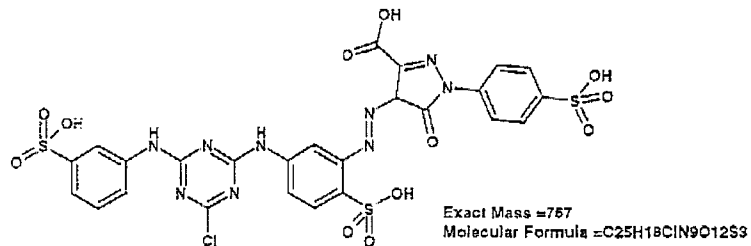
- 1) Zusammenstellung der Ergebnisse
- 2) UV-Chromatogramm
- 3) ESI-Spektren

Problemstellung / Thema:
Verunreinigungen in Procion P 4G

Untersuchungsverfahren: HPLC-(UV, ESI)-MS am HP1100/ LC-Q
Säule: 125x2mm Superspher60 RP-SelectB, R028
Eluent: A:Wasser + 5 mM Ammoniumacetat (pH8 mit NH₄OH) B:ACN
Gradient: 0% B (5 min) – 10 min – 40% B, 0.3 mL/min, 40°C
Detektion: ESI pos. Ionen, DAD 268/ 408 nm

Ergebnisse:
Produkt P-4G – Die HPLC-Trennung wurde analog den Bedingungen aus früheren Untersuchungen (Befund 2001-10708) durchgeführt. Das UV-Chromatogramm (268 nm) der Probe ist der Anlage 1 zu entnehmen. Anhand der ESI-Spektren wurden folgende Strukturvorschläge zugeordnet (vgl. Anlage 2):

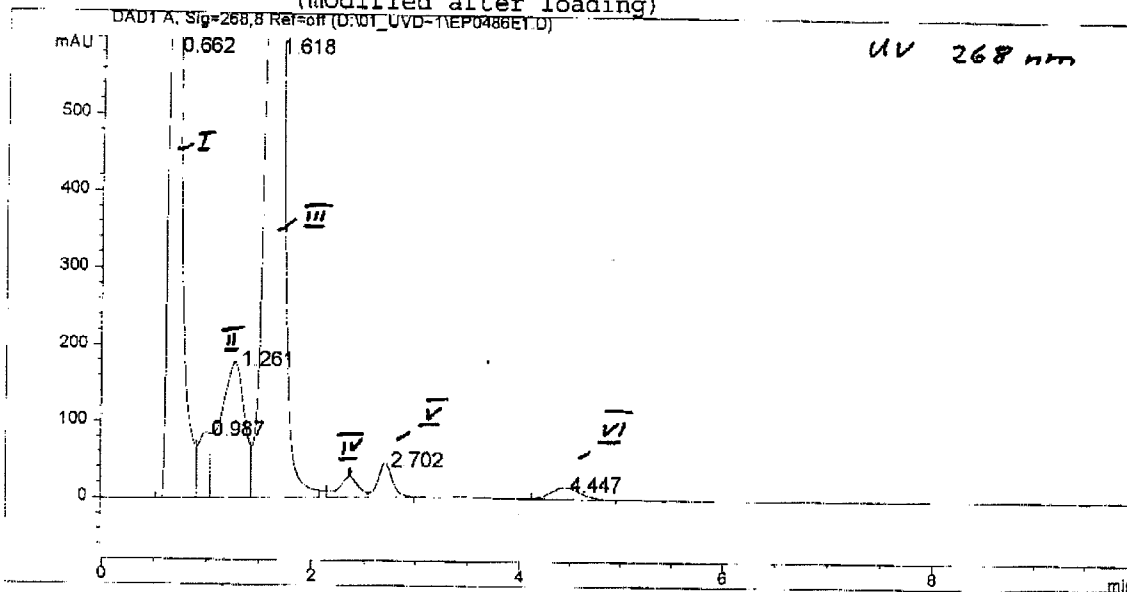
Peak Nr.	MG	Strukturvorschläge
I	720	---
II	904	---




```

=====
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Sample Name     : Produkt P4G
Acq. Operator   : mh
Vial            : 1
Inj Volume      : 0.5 µl

Acq. Method     : D:\HPCHEM\1\METHODS\AK_STD.M
Last changed    : 7/9/02 1:10:33 PM by mh
                  (modified after loading)
Analysis Method : C:\HPCHEM\1\METHODS\AK_STD.M
Last changed    : 7/19/02 10:59:53 AM
                  (modified after loading)
    
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Area Percent Report

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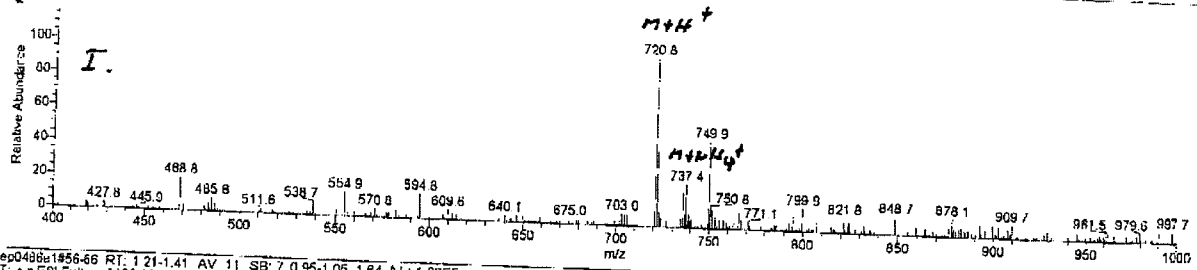
Sorted By      : Signal
Multiplier     : 1.0000
Dilution       : 1.0000
    
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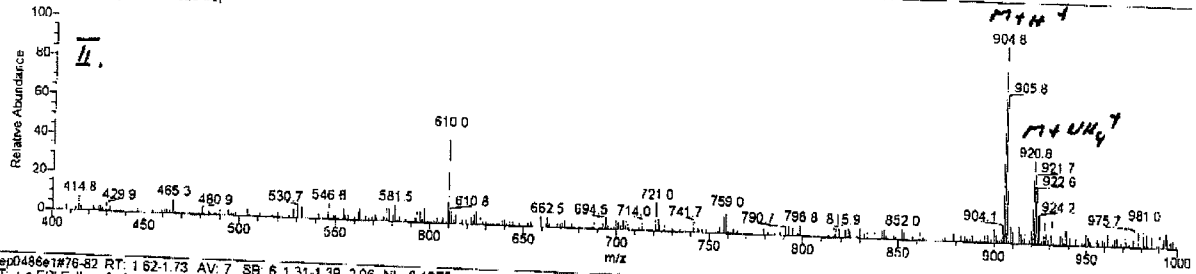
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2	0.987	VV	0.1090	638.63397	85.67457	2.2142
3	1.261	VV	0.2274	2859.45923	178.02333	9.9139
4	1.618	VB	0.1496	1.54487e4	1586.94299	53.5618
5	2.357	BV	0.1885	352.10153	27.29906	1.2208
6	2.702	VB	0.1442	430.02368	44.73168	1.4909
7	4.447	BB	0.3155	309.49847	15.07915	1.0731

Totals : 2.88428e4 3251.86419

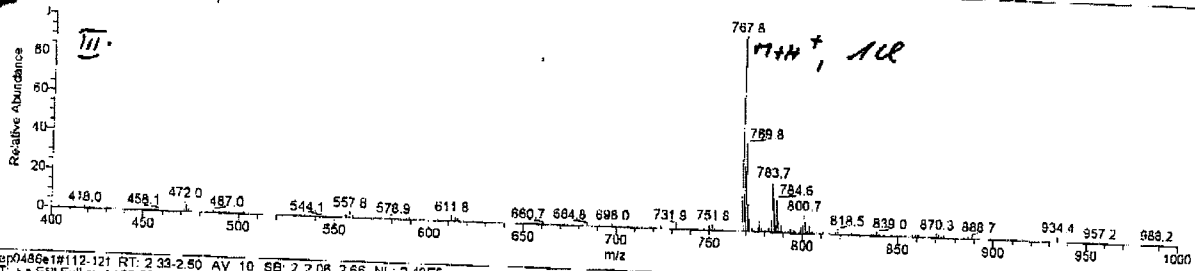
*** End of Report ***



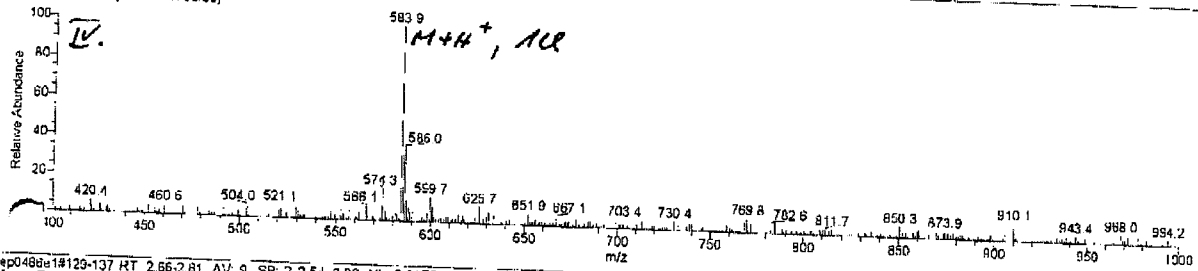
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T: + c ESI Full ms [130 00-1000 00]



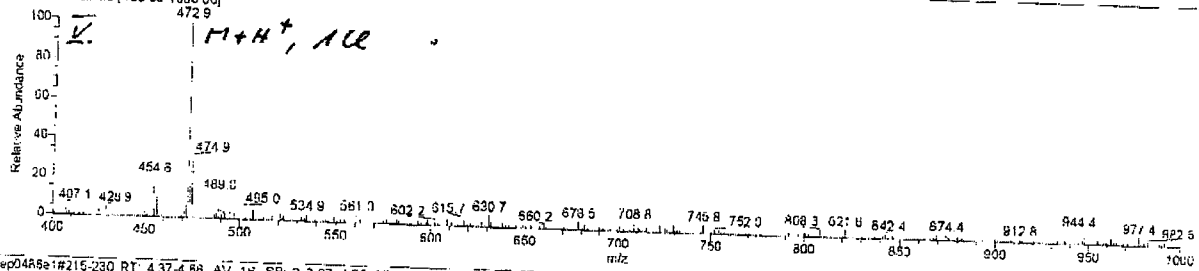
ep0486e1#76-82 RT: 1.62-1.73 AV: 7 SB: 6 1 31-1 39 2 06 NL: 8 10E5
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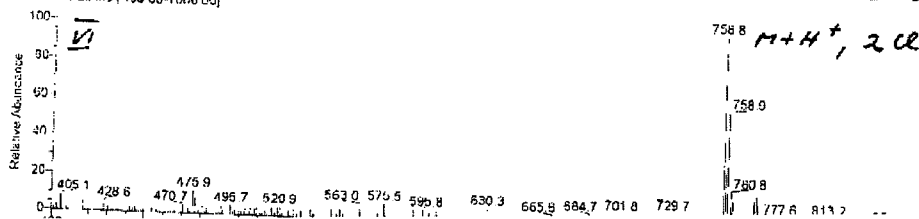
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ep0486e1#215-230 RT: 4.37-4.88 AV: 16 SB: 2 3 07 4 93 NL: 1 92E5
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BASF -Reaktivfarbstoff / Produkt P-4G

Methode : F:\CLASS-VP\Methods\Triphenioxazine\Nitrosulfosäure und Aminosulfosäure
ED\Vorprodukte TPD Base.met

Datei : F:\HPLC-Daten\Farbstoffe\BASF\BASF Synergy TBAHS pH7 0,7 ml 001a

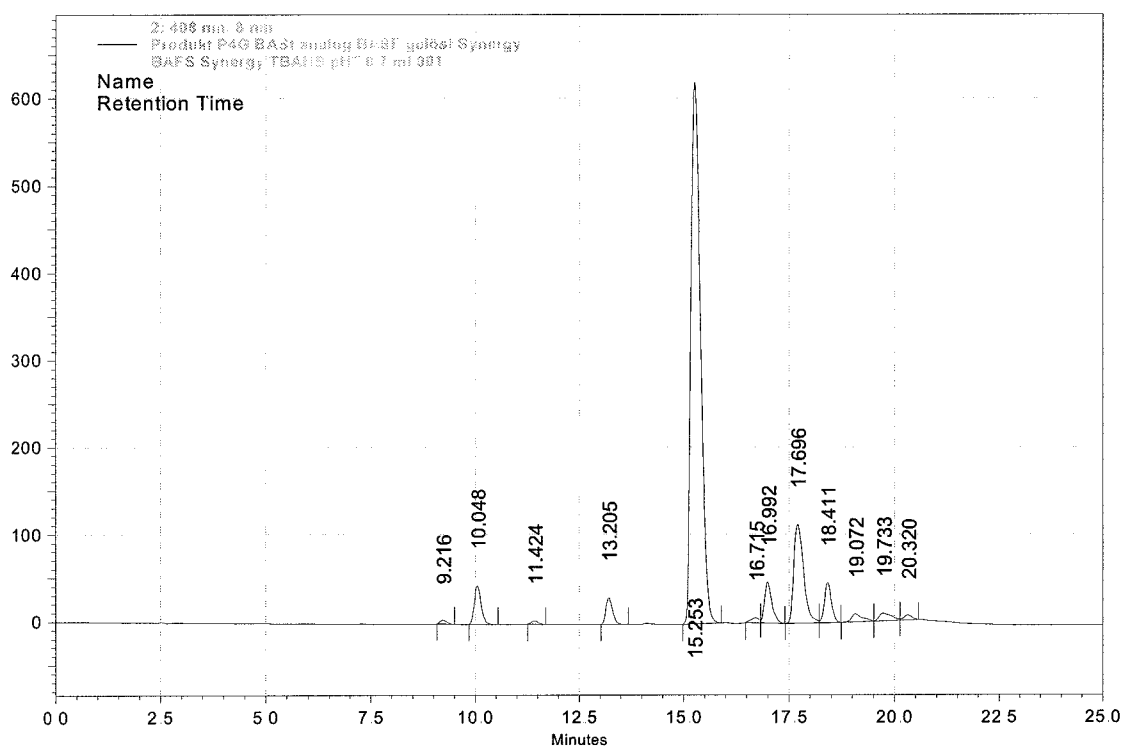
Probenbezeichnung : Produkt P4G BASF

Säule : Phenomenex Synergy 4µ Hydro-RP80A 150*4,6 mm

Laufzeit : 14.03.06 10:14:27

Einwaage : 94 mg Fluss : 0,7 ml/min

HPLC bei 408 nm:



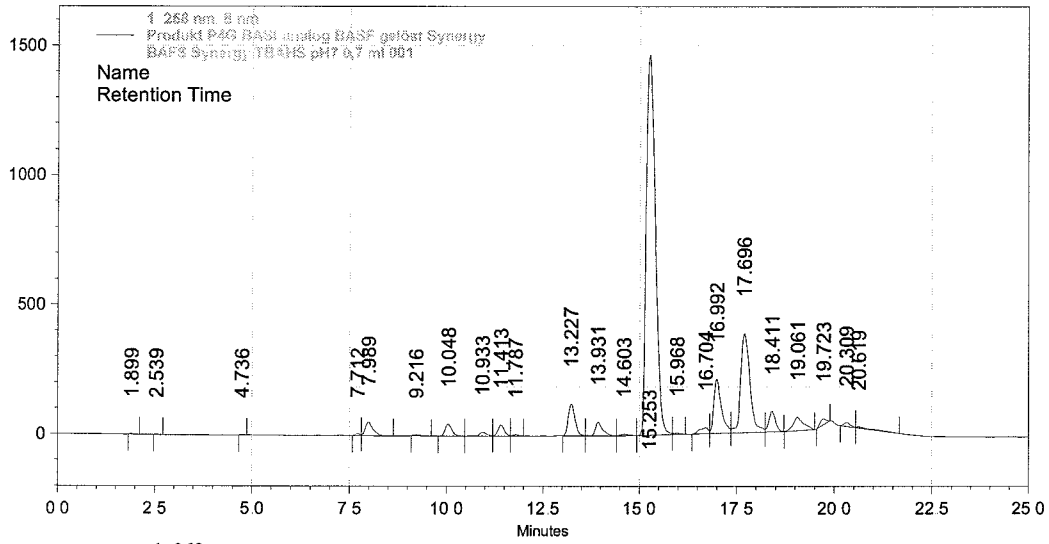
2: 408

nm, 8 nm

Pk #	Name	Retention Time	Area	Area Percent
1		9.216	45280	0.32
2		10.048	507271	3.55
3		11.424	41558	0.29
4		13.205	336180	2.35
5	Hauptkomponente P-4G	15.253	9943861	69.65
6		16.715	73551	0.52
7		16.992	567177	3.97
8	Größte NK	17.696	1815576	12.72
9		18.411	538225	3.77
10		19.072	161023	1.13
11		19.733	174525	1.22
12		20.320	72202	0.51

Totals			14276429	100.00
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HPLC bei 268 nm:



1: 268

nm, 8 nm

Pk #	Name	Retention Time	Area	Area Percent
1		1.899	11458	0.03
2		2.539	7260	0.02
3		4.736	15828	0.04
4		7.712	60466	0.15
5		7.989	715750	1.76
6		9.216	42971	0.11
7		10.048	551745	1.35
8		10.933	195504	0.48
9		11.413	481513	1.18
10		11.787	55299	0.14
11		13.227	1488784	3.65
12		13.931	688770	1.69
13		14.603	84404	0.21
14	Hauptkomponente P-4G	15.253	24177492	59.32
15		15.968	47678	0.12
16		16.704	389421	0.96
17		16.992	2813661	6.90
18	Größte NK	17.696	6379208	15.65
19		18.411	958123	2.35
20		19.061	979027	2.40
21		19.723	250447	0.61
22		20.309	168009	0.41
23		20.619	125207	0.31
24		26.357	70501	0.17

Totals				
		40758526	100.00	0.000