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B99-73

Study Title

Validation of BASF Technical Procedure 354/2: Determination of BF 490-1, BF 490-2 and BF 490-9 in Fat, Muscle, Liver, and Kidney of Beef

EPA Guideline Number

171-4

Authors

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A.J. Krotsky

Study Completed On

September 1996

Performing Laboratories

BASF Aktiengesellschaft
Ecology and Environmental Analytics
D-67114 Limburgerhof, FRG

Lab Method Number: Method 354/2

BASF Registration Document No.

96 / 10625

This report consists of 92 pages

EAC METHOD # : 3571Z

PR 86-5 DATA CONFIDENTIALITY CLAIM

No claim is made for any information contained in this study on the basis of its falling within the scope of FIFRA 10 (d) (1) (A), (B), or (C).

COMPANY BASF CORPORATION / AGRICULTURAL PRODUCTS GROUP

COMPANY AGENT Karen Blundell **DATE** Sept. 12, 1996

Senior Registration Specialist — Karen R Blundell

Title **Signature**

96/10625 002

LAB METHOD #: METHOD 354/2

GOOD LABORATORY PRACTICES STATEMENT

This study meets the requirements for 40 CFR 160, Good Laboratory Practices, but differs in the following manner:

This study was conducted in accordance with the GLP Regulations; Appendix 1 to Part 19a, Section 1, Chemikaliengesetz of 25 July 94 (Official bulletin/Federal Republic of Germany, 1994, pg. 1703).

Some data sets were not conducted under GLP Regulations and are designated as such in the report.

BASF Corporation
Agricultural Products Group

Submitter and Sponsor: Karen R Blundell
Karen Blundell
Senior Registration Specialist

Date: Sept. 25, 1996

Study Director Signature: see page 3

RheinlandPfalz

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Mainz, 20. Dezember 1994

G L P - B E S C H E I N I G U N G

Bescheinigung

Hiermit wird bestätigt, daß die Prüfeinrichtung

APS/U
der Firma BASF AG
- Landwirtschaftliche Versuchsstation -

67114 Limburgerhof

Bundesrepublik Deutschland

am 01. und 14. September 1993 und vom 15.-16. Dezember 1993 von der für die Überwachung zuständigen Behörde über die Einhaltung der Grundsätze der Guten Laborpraxis inspiziert worden ist.

Es wird hiermit bestätigt, daß die Prüfungen in dieser Prüfeinrichtung nach den Grundsätzen der Guten Laborpraxis durchgeführt werden.

Im Auftrag

Dr. Fresenius

Certificate

It is hereby certified that the test facility

APS/U
der Firma BASF AG
- Landwirtschaftliche Versuchsstation -

67114 Limburgerhof

Federal Republik of Germany

at 01. and 14. September 1993 and from 15.-16. December 1993 was inspected by the competent authority regarding compliance with the principles of Good Laboratory Practice.

It is hereby certified that studies in this test facility is conducted in compliance with den principles of Good Laboratory Practice.

Anlage zur GLP-Bescheinigung für die Prüfeinrichtung APS/U

Die Prüfeinrichtung führt in Übereinstimmung mit dem GLP-Grundsätzen Prüfungen in folgenden Prüfkategorien durch:

1. Prüfungen auf physikalisch-chemische Eigenschaften und Gehaltsbestimmungen
4. Umwelttoxikologische Prüfungen zu Auswirkungen auf aquatische und terrestrische Organismen
5. Prüfungen zum Verhalten im Boden, Wasser und in der Luft; Bioakkumulation; Metabolismus
6. Prüfungen auf Rückstände
7. Prüfungen zu Auswirkungen auf Mesokosmen und natürliche Ökosysteme
8. Analytische Untersuchungen an biologischen Materialien

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE

The study was conducted in compliance with Good Laboratory Practice Regulations; Appendix 1 to § 19a, Section 1, Chemikaliengesetz of 25 July 94 (Official Bulletin/Federal Republic of Germany I, 1994, P. 1703).

Study Director: Dr. U. Rabe

Date: 12. 09. 96 Signature: U. Rabe

B A S F Aktiengesellschaft

BASF Landw. Versuchsstation - Postfach 120 - 67114 Limburgerhof

STATEMENT OF THE QUALITY ASSURANCE UNIT

Study Code: METHOD 354/2

The quality assurance unit of the testing facility inspected the study plan and audited the final report. Findings were reported to the study director and to management.

Date of inspections	Report to study director and to management
26-Apr-1994	
11-Sep-1996	

67114 Limburgerhof

Schepers 12.9.96
.....
Signature QAU

Quality Assurance Statement

We hereby attest to the authenticity of the study and guarantee that the data are correct and accurate to the best of our knowledge and that the study was performed by the procedures described.

Study Director:

Dr. U. Rabe
APS/UP
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Date: 12.9.96

Signature: Mackenroth

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Date: 11.09.96

Signature:

ABSTRACT

BASF Method 354/2 was developed as individual residue method for the analysis of BF 490-1, BF 490-2 and BF 490-9 in skeletal muscle, peritoneal fat, liver and kidney of beef.

The analytes are extracted from a 25 g sample aliquot into 300 ml methanol. The clean-up of the extracts involves $Ca(OH)_2$-precipitation, phase partitioning into DCM and SPE NH_2-column separation step. It is followed by further clean-up with preparative HPLC on a C_{18}-column.

The analytes are quantified by analytical HPLC with column switching on a NH_2-/CN-column combination and UV-detection at 270 nm. BF 490-1, BF 490-2 and BF 490-9 are determined as intact, compounds. Alternatively and as confirmatory technique the analytes can be quantified by LC/MS on an analytical HPLC with NH_2-column.

The analysis of a set of 10 samples requires about 20 hours of work.

The method has a limit of quantitation of 0.01 mg/kg for all analytes in all tissues tested. The limit of detection of method 354/2 is approximately 0.002 mg/kg for all analytes in all tissues.

In in-house and external validation studies performed at fortification levels of 0.01 and 1.00 mg/kg the overall recoveries for all analytes in all matrices of the method were 89.8 % (+/- 9.0 %) at a level of fortification of 0.01 mg/kg and of 80.7 % (+/- 2.1 %) at a level of fortification of 1.00 mg/kg.

The overall repeatability of BASF method 354/2 at a level of fortification of 0.01 mg/kg was 0.003 mg/kg and 0.112 mg/kg at a level of fortification of 1.00 mg/kg. The reproducibility was tested in-house and in an external laboratory. The level of reproducibility was 0.001 mg/kg at 0.01 mg/kg fortification level and 0.080 mg/kg at 1.00 mg/kg fortification level.

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Commencement of laboratory operations	April 1994
Completion of laboratory operations	September 1994
Completion of the final report	September 1996

B Guidelines and Compliance

Test Method

- Identity

Residue Analysis of Crop Protectants, Announcement VI of the Senat Commission for Crop Protectants, Method Collecting of the working group "Analytics", German Research Organisation, V and XI, 1991

Criteria for assessment of plant protection.products in the registration procedure, 3. Residue Analysis, BBA, 1993

EPA Pesticide Assessment Guidelines, Subdivision O, 171-4

- Justification

Validation of BASF Method 354/2

- Copy of Method

Not applicable

- Choice of test method

Not applicable

- Deviations from test method

Not applicable

Good Laboratory Practice (GLP)

The study was conducted in compliance with, and by a facility certified as to its competence to conduct studies in compliance with the principles of GLP.

Certifying authority

Ministerium für Arbeit, Soziales und Gesundheit (Ministry for Labour, Social Affairs and Health), Baustraße 9, 55116 Mainz

GLP-Guidelines covered

Appendix 1 to § 19 a, Section 1, Chemikaliengesetz of 25th July 1994 (Official Bulletin/-Federal Republic of Germany I, 1994, p. 1703).

GLP Consensus Document: The Application of the GLP Principles to Field Studies, Env. Monograph No. 50, OECD, Paris 1992.

EC Commission Guidelines

The study was conducted in compliance with Annex II to Directive 91/414/EEC.

C Technical Information

Performing Laboratory

All parts of this study were conducted by BASF Aktiengesellschaft, Ecology and Environmental Analytics, Plant Metabolism and Residue Studies, Postfach 120, D-67114 Limburgerhof.

Location of raw data

Archive belonging to APS/U, building Li 444

Filing of reports, raw data and samples

Study plan, raw data, original report or certified duplicates, specimens and samples, will be stored in an archive at BASF Aktiengesellschaft, Landwirtschaftliche Versuchsstation, D-67117 Limburgerhof, at least for the period of time specified in the GLP regulations.

Technical personnel

Laboratory

Werner Dams
Zbigniew Schweda

Statistical Methods used

Normal calculations for the determination of mean values, standard deviations (SD) and of the coefficients of variation (CV).

Reference to Related Studies

Validation of the Methods of Analysis for BAS 490 F Metabolites in Milk and Tissue from Dairy Cows, Study Code BSF 528, Huntingdon Research Centre, UK, 13 December 1994.

1. INTRODUCTION

BAS 490 F is a new Strobilurin type fungicide used in cereals, apples, citrus and vine against mildew and rust. Metabolism investigations in goat (ref. 5) identified three relevant metabolites for analysis as listed below:

Tissue	Analytes to be quantified
Muscle	BF 490-1 and BF 490-2
Fat	BF 490-1, BF 490-2, BF 490-9
Kidney	BF 490-1, BF 490-2, BF 490-9
Liver	BF 490-1 and BF 490-9

2. PRINCIPLE OF THE METHOD

For the extraction of the metabolites 25 g of the sample material are homogenized in methanol. The resulting homogenates are cleared from solids by filtration and the cleared extracts are reduced to the aqueous phase by rotary evaporation. After addition of methanol interfering, non-polar compounds are carrier-precipitated by the addition of solid $Ca(OH)_2$. The cleared supernatant is acidified, reduced to the aqueous phase and supplemented with ascorbic acid and a phosphate buffer. This acidic extract is phase partitioned into dichloromethane. The dichloromethane phase is further purified and concentrated on a SPE-NH_2-column. Ascorbic acid present in the eluate is eliminated by a second phase partitioning step with dichloromethane at low pH. The metabolites are separated from matrix interferences by preparative HPLC on a RP-C_{18}-column and collected in appropriate fractions. Final quantification of individual analyte concentrations is performed by analytical HPLC on a NH_2-/CN-column combination with column-switching. The analytes are detected with an UV detector at 270 nm.

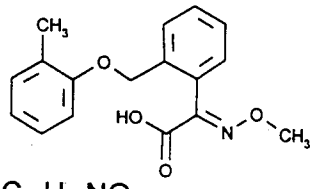
3. TEST AND REFERENCE SUBSTANCES

3.1 Specifications

Metabolite BF 490-1

Common name: not issued
Chemical name: 2-[o-(o-methylphenoxymethyl)phenyl]-2-(methoxyimino) acetic acid
Reg. No.: 262 451

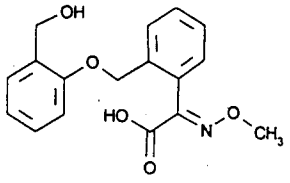
Structural formula:



Chemical formula: $C_{17}H_{17}NO_4$
Molecular weight: 299.33
Study No.: PCP 02534
Purity: 98.5%
Stability: 2 years, until 05/95

Metabolite BF 490-2

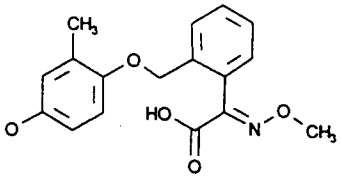
Common name: not issued
Chemical name: 2-[o-(o-hydroxymethylphenoxymethyl)phenyl]-2-(methoxyimino) acetic acid
Reg. No.: 291 685
Structural formula:



Chemical formula: $C_{17}H_{17}NO_5$
Molecular weight: 315.33
Study No.: PCP 02910
Purity: 97.7%
Stability: not available

Metabolite BF 490-9

Common name: not issued
Chemical name: 2-[o-(p-hydroxy-o-methylphenoxymethyl)phenyl]-2-(methoxy-imino) acetic acid
Reg. No.: 292 932
Structural formula:



Chemical formula: $C_{17}H_{17}NO_5$
Molecular weight: 315.33
Study No.: PCP 02271
Purity: 99.8%
Stability: 2 years, until, 11/94

3.2 Stability of Reference Solutions

BF 490-1

Stability: Stable for 91 days at +4°C in acetonitrile in the dark
Study No.: SST-490/1-92 (ref. 2)

BF 490-2 and BF 490-9

Stability: Stable for 60 days at +4°C in acetonitrile in the dark
Study No.: SST/001/94 (ref. 3)

4. MATERIALS AND METHODS

Note:
The materials, chemicals and the equipment specified below were used for the validation studies in chapter 5. They are specified as examples only and may be substituted by supplies with similar specifications. If the use of supplies other than those stated is intended, applicability to this method must be confirmed prior to method validation and/or routine analysis.

4.1 Equipment for extraction and sample clean-up

Wide neck powder bottle for sample extraction	(500 ml)
Separatory funnels	(50 ml, 100 ml, 250 ml)
Round bottom flasks	(1 l, 250 ml, 500 ml)
Measuring flasks	(100 ml, 10 ml, 5 ml)
Conical flasks	(10 ml, 50 ml)
Volumetric flasks	(10 ml)
Volumetric pipettes	(glass, 1, 5, 10 ml)
Measuring pipettes	(glass, 1, 5, 10 ml)
Homogenizer (Polytron 3035 or Ultra turrax)	
Glass funnels	(9 cm diameter)
Black ribbon filters	(9 cm diameter)
Vacuum pump incl. vacuum controler	
Rotary evaporator equipped with heating water bath vacuum- and thermo controller	
Ultrasonic water bath	
Micro pipettes, appropriate pipette tips	(100 µl or equiv.vol. range),
BAKER 10 extraction system complete with taps and vacuum controller	J.T. Baker, Philipsburg, N.Y.
Sample collector rack	J.T. Baker
Adaptors for extraction system	J.T. Baker
Empty, disposable BAKER columns (plastic, 6 ml)	J.T. Baker
Reservoirs (15 ml) for Baker columns	J.T. Baker
Bulk NH_2- chromatography packing material for BAKER columns	J.T. Baker
Cotton wool	prewashed with DCM and dried at 30°C

Preparation and Preconditioning of BAKER NH_2-columns:

Empty, disposable BAKER columns (6 ml) are filled with 1.5 g each of the bulk NH_2-material specified above. The packing material is covered with the frit supplied with the column. For preconditioning the column is washed once with 5 ml of methanol, followed by 5 ml of an aqueous solution of KH_2PO_4 (0.1 M, pH 7.5). The column is rinsed with 5 ml ddH_2O and 5 ml methanol. The column is completely dried under vacuum for a short while and finally rinsed with 5 ml dichloromethane.

4.2 Reagents

4.2.1 Chemicals

Note:
All chemicals used must be at least of "analytical grade" or must meet equivalent specifications.

Ethyl acetate	
Methyl-tert.butylether (MTBE)	
Methanol	
Dichloromethane (DCM)	
Acetonitrile	
Formic acid (conc.)	
Phosphoric acid (85 %)	
Ascorbic acid	
HCl	
NaCl	
Na_2SO_4	
KH_2PO_4	
$Ca(OH)_2$	
Water (Milli-Q-Grade or equivalent)	*in this method referred to as ddH_2O*

4.2.2 Solvent Mixtures and Buffers

Note:
The coding system in the brackets (SM I) is used to identify the solvent mixtures in chapter 5. Analytical procedure.

"Acidic" water, pH 2 (SM I):
1 ml of 10 M HCl are added to 999 ml ddH_2O (pH 2)

Acidic phosphate-ascorbic acid buffer (SM II):
50 g of KH_2PO_4 and 50 g of ascorbic acid are filled up to approximately 450 ml. The solution is adjusted to pH 2 with conc. formic acid and subsequently made up to 500 ml with ddH_2O to yield a final concentration of 0.1 g/ml of each compound (approx. pH 2.0)

Alkaline phosphate-ascorbic acid buffer (SM X):
50 g of KH_2PO_4 and 50 g of ascorbic acid are filled up to approximately 450 ml. The

solution is adjusted to approximately pH 10 with 1 N NaOH-solution and subsequently made up to 500 ml with ddH_2O to yield a final concentration of 0.1 g/ml of each compound (approx. pH 9.5).

Ethyl acetate/Methyl-tert.-butylether mixture (SM III)
Ethylacetate and methyl-tert.-butylether are mixed in a volume ratio of 1 + 1.

Phosphate buffer (SM IV)
13.6 g of KH_2PO_4 are made up to approximately 900 ml. The solution is adjusted to approximately pH 7.5 and subsequently made up to 1000 ml with ddH_2O to yield a final concentration of 0.1 M (approx. pH 7.5)

Note:
For storage the buffers SM II, SM X and SM IV must be kept refrigerated.

4.2.3 Solutions for BAKER SPE-columns

NH_2-column elution mixture (SM V):
Contains 350 ml aqueous NaCl solution (ddH_2O saturated with NaCl) + 7 ml formic acid (conc.) + 10.5 g ascorbic acid + 100 ml Acetonitrile + 50 ml methanol.

This solution must be prepared minimum 24 h prior to use. After precipitation of NaCl the superantant should be cleared by filtration or decantation before use in the method.

4.2.4 Stock and Spiking solutions

Individual stock solutions of BF 490-1, BF 490-2 or BF 490-9 in concentrations of 1 mg/ml each are prepared in acetonitrile (for standard stability see 3.1 and 3.2).

From these stock solutions spiking solutions in acetonitrile are prepared with analyte concentrations of 0.10 and 10.0 µg/ml. For fortification experiments the analytes may be combined in one spiking solution with individual analyte concentrations specified above.

4.2.5 Standard solutions for calibration

The stock solutions specified in 4.2.4 may be used for the preparation of calibration standards.

From the 1 mg/ml stock solutions in acetonitrile calibration standards are prepared in a mixture of acetonitrile (1 volume part) + "acidic" water (SM I) (9 volume parts) with analyte concentrations in the range of ~~125 to 500 ng/ml~~ (or higher if required). Calibration standards may contain individual analytes or a combination of the metabolites under investigation.

4.2.6 Buffers for Preparative HPLC Chromatography

Preparative HPLC elution buffer A (SM VI)
Contains ddH_2O + acetonitrile + formic acid (conc.) 90 + 10 + 0.5 (v/v/v).

Preparative HPLC elution buffer B (SM VII):
Contains ddH_2O + acetonitrile + formic acid (conc.) 10 + 90 + 0.5 (v/v/v).

4.2.7 Buffers for Analytical HPLC Chromatography

Analytical NH_2-HPLC column:
Amino-HPLC elution buffer (SM VIII):
Contains ddH_2O + acetonitrile + formic acid (conc.) 90 + 10 + 1 (v/v/v).

The peformance of HPLC NH_2-columns strongly depends on the activity of the carrier material and, to a minor degree, the "age" and "use" of the column. To account for these differences, the concentration of the formic acid in the buffer must be optimized for each column, especially if a new column is used. For new columns or highly activated columns, the concentration of formic acid in the buffer may be increased to 1.5 %. Extensive use of NH_2-columns may require to decrease the formic acid concentration to minimum 0.5 % (see also chapter 5.9).

Analytical CN-HPLC column:
CN-HPLC elution buffer A (SM IX):
Contains ddH_2O + acetonitrile + phosphoric acid (85 %) 90 + 10 + 0.25 (v/v/v).
CN-HPLC elution buffer B (SM X):
Contains ddH_2O + acetonitrile + phosphoric acid (85 %) 55 + 45 + 0.25 (v/v/v).

4.3 HPLC Instrumentation and Chromatography Conditions

4.3.1 Preparative HPLC

2 HPLC - pumps	Model 422, Kontron
HPLC-Detector	Model 430, Kontron
Autosampler	Model 460, Kontron, with 2000 µl loop
Program controller	DATA System 450-MT2 V3.90, Kontron
Fraction collector	

Preparative column specification:
Reversed phase, 120-5-C_{18}-Nucleosil, 250 mm x 8.0 mm (diam.), TPC-Klaus Ziemer GmbH, Mannheim, FRG

Precolumn specification:
Reversed phase, 120-5-C_{18}-Nucleosil, 50 mm x 10 mm (diam.), TPC-Klaus Ziemer GmbH, Mannheim, FRG

4.3.2 Analytical HPLC

2 HPLC - pumps	Model 420, Kontron
HPLC-Detector	Model 430, Kontron
Autosampler	Model 460, Kontron, with 500 µl loop
Program controller	DATA System 450-MT2 V3.90, Kontron
Switching valves	Rheodyne

Amino-column specification:
Nucleosil-NH_2-120-5, 125 mm x 4.0 mm (i.d.), Bischoff-Analysentechnik und Geräte GmbH, Leonberg, FRG

Analytical Cyano (CN-) column specification:
Nucleosil-120-7-CN, No. 6 (7 µm), 250 mm x 4.0 mm (i.d.), Macherey & Nagel GmbH, FRG, Art. No. 720 057

Note:
The equipment listed in 4.3.1 and 4.3.2 was used for the validation of the method in muscle. It is representative for the validations in liver, kidney and fat, too. It may be substituted, however, by equipment with similar specifications.

4.3.3 HPLC Instrumentation Testing

It is advisable to verify the retention times of the analytes on the preparative C_{18}-column as well as on the NH_2-HPLC-column prior to each analytical series in order to facilitate the quantitative analyte transfer during fraction collection as well as column switching. For this, appropriate standard solutions can be injected into the system to verify the peak retention times of BF 490-1, BF 490-2 and BF 490-9 on both columns by direct monitoring with an appropriate detector.

Preparative HPLC-instrumentation:

BF 490-1 and BF 490-2 (for muscle) or BF 490-1, BF 490-2 and BF 490-9 (for kidney) and BF 490-1 and BF 490-9 (for liver) are dissolved in a solvent mixture of 15 % acetonitrile in "acidic water" at a final concentration of 1 µg/ml of each of the analytes.

Analytical NH_2- column:

The calibration standards described in 4.2.5 may be used for the verification of the column switching times. The analytes should be used at a concentration of 1 µg/ml.

For retention times of the analytes on both columns refer to chapter 5.7 of this report.

4.3.4 Mode of Detection

The analytes are detected by UV-monitoring at a wavelength of 270 nm.

5. ANALYTICAL PROCEDURE

5.1 Spiking of Samples for Recovery Experiments

25 g of untreated sample material are weighed into a wide neck powder bottle (glass, 500 ml). 2.5 ml of each of the spiking solutions with analyte concentrations of 0.1 and 10.0 µg/ml (see 4.2.4) are added to the samples. The correlation between the concentration of the spiking solution and the resulting final analyte concentration in the sample is shown below:

Sample Weight	Concentration of spiking solution	Volume of spiking solution	Level of Fortification
25 g	0.1 µg/ml	2.5 ml	0.010 mg/kg (*)
25 g	10.0 µg/ml	2.5 ml	1.000 mg/kg

(*) Proposed limit of determination of the method.

5.2 Extraction of the Sample Material

25 g of sample material are extracted with 300 ml of methanol by homogenisation for 5 minutes using a Polytron at 6000 RPM. The homogenate is suction filtrated under vacuum through a black ribbon filter. The residual solids in the filter are washed with two volumes of 50 ml each of methanol which are added to the first filtrate. The combined filtrates are transferred into a preweighed one liter round bottom flask.

The methanol is removed from the extract using a rotary evaporator at 250 mbar reduced pressure and a water bath temperature of approximately 60°C. This reduction step should be continued until only an oily residue is left in the flask (approximate weight of 5 - 10 g). The flask is filled up with ddH_2O up to a volume of 100ml and then 50 ml of methanol is added.

5.3 Carrier Precipitation with $Ca(OH)_2$

To the aqueous methanol extract (from 5.2) 5 g of solid $Ca(OH)_2$ are added. The suspension is vigorously shaken for approximately 1 minute and allowed to settle (about 45 to 60 minutes). The suspension is filtered through a folded filter and the clear filtrate collected in a 250 ml measuring cylinder.

5.4 Phase Partitioning with Dichloromethane

A 125 ml aliquot of the filtrate from 5.3 is transferred into a preweighed 500 ml round bottom flask and acidified with 4 ml of concentrated formic acid. Using a rotary evaporator, the filtrate is subsequently reduced to approximately 5 to 10 g. To this aqueous phase 5 ml of the acidic phosphate-ascorbic acid buffer (SM II) are added. The pH of the resulting solution is adjusted to 2 - 2.5 with conc. formic acid. The total weight is adjusted to exactly 20 g with ddH_2O.

flask. These pooled dichloromethane phases are subsequently reduced to dryness by rotary evaporation (vacuum 600 mbar) and a water bath temperature of 60°C. The dry residue is redissolved in 2 ml dichloromethane and transferred into a 10 ml conical flask. The round bottom flask is rinsed with two more volumes of 2 ml dichloromethane which are added to the conical flask, too. These pooled dichloromethane phases are subsequently reduced to dryness by rotary evaporation (vacuum 600 mbar) and a water bath temperature of 60°C. Residual vapours of dichloromethane are removed by flushing the conical flask with clean air.

5.7 Clean-up of the Metabolites by Preparative HPLC

The dry residue from 5.6 is redissolved in 450 µl acetonitrile with the aid of an ultrasonic waterbath. 2.55 ml of the "acidic ddH_2O" (SM I) are added to the extract to yield a total volume of 3 ml. For the fractionisation of the metabolites 1 ml of this extract solution is injected into the preparative HPLC.

Chromatography Conditions

The HPLC is operated at a total buffer flow rate of 3 ml/min.

The solvent gradients and the time windows for the collection of metabolite containing fractions are specified below for muscle, fat, liver and kidney. They were used for the in-house validations at BASF but are given as examples only.

The retention time of the analytes may vary with the type of sample material. In addition and as outlined in chapter 4.3.3 the retention times also depend on the type and performance of the HPLC instrumentation. Therefore it is essential to test the performance of the preparative HPLC set-up in use with the sample material under investigation. If necessary, either the solvent gradients or "metabolite collection windows" may be adapted the HPLC in use.

Note:
As is common practise with HLPC analysis, it is advisable to "stabilize" the on-column retention times of the metabolites before Injecting the first sample of an analytical series. For this the entire solvent gradient should be pre-run using extract from an untreated sample.

Routine analysis with method 354/2 has shown that metabolite retention times on the preparative HPLC may differ about +/- 2 minutes from the times given above if different HPLC systems and columns from different suppliers were used. However, within each instrumentation, the run-to-run variation of the retention times is very small (< 20 seconds).

<u>On-column conditions for muscle:</u>

The metabolites BF 490-1 and BF 490-2 are fractionated from the preparative column with the gradient elution profile of the HPLC buffers A (SM VI) and B (SM VII) as outlined below:

The buffer gradients and collection times were applied to the GLP-validation, the results of which are shown in chapter 11.1.1.

Time (minutes)	Buffer A (Vol. %)	Buffer B (Vol. %)	Typ of Gradient	Fractions collected	Date: 26.7.94
00.00	100	0	start		
19.20				I, on	
20.55				I, off	
22.00	35	65	linear		
24.00	0	100	linear		
24.85				II, on	
26.10				II, off	
27.00	0	100			
27.50	100	0			
36.0	100	0			

Analyte retention times:

Applying the chromatography conditions outlined above, typical peak retention times of the analytes are for:

BF 490-2: approximately 19.5 minutes (fraction I)
BF 490-1: approximately 25.0 minutes (fraction II)

On-column conditions for kidney and fat:

The metabolites BF 490-1, BF 490-2 and BF 490-9 are separated on and fractionated from the preparative column with the gradient elution profile of HPLC buffers A (SM VI) and B (SM VII) as outlined below:

An example of the buffer gradients and collection times were applied to the GLP-validation, the results of which are shown in chapters 11.1.2 and 11.1.3.

Time (minutes)	Buffer A (Vol. %)	Buffer B (Vol. %)	Typ of Gradient	Fraction collected
00.00	100	0	start	
18.05				I, on
19.90				I, off
22.00	35	65	linear	
24.00	0	100	linear	
24.15				II, on
25.24				II, off
27.00	0	100		
27.50	100	0		
36.0	100	0		

Analyte retention times:

Applying the chromatography conditions outlined above, typical peak retention times of the analytes are for:

BF 490-2: approximately 18.0 minutes (fraction I)
BF 490-9: approximately 19.7 minutes (fraction I)
BF 490-1: approximately 25.0 minutes (fraction II)

All analyte containing fractions are collected in test tubes or other appropriate vessels.

On-column conditions for liver:

The metabolites BF 490-1 and BF 490-9 are separated on and fractionated from the preparative column with the gradient elution profile of HPLC buffers A (SM VI) and B (SM VII) as outlined below:

The buffer gradients and collection times were applied to the GLP-validation, the results of which are shown in chapter 11.1.4.

Time (minutes)	**Buffer A (Vol. %)**	**Buffer B (Vol. %)**	**Typ of Gradient**	**Fraction collected**	**Date: 4.7.94 "Non-GLP"**
00.00	100	0	start		
20.10				I, on	
21.30				I, off	
22.00	35	65	linear		
24.00	0	100	linear		
24.80				II, on	
26.05				II, off	
27.00	0	100			
27.50	100	0			
36.0	100	0			

Analyte retention times:
Applying the chromatography conditions outlined above, typical peak retention times of the analytes are for:

BF 490-9: approximately 19.5 minutes (fraction I)
BF 490-1: approximately 25.0 minutes (fraction II)

All analyte containing fractions are collected in test tubes or other appropriate vessels.

5.8 Preparation of the Injection Sample for Analytical HPLC

The fractions from 5.7 which contain the analytes are transferred into 10 ml conical flasks. The collection containers are rinsed twice with 1 ml acetonitrile each which are also added to the conical flask. The solvents are subsequently reduced to dryness with an N-EVAP and a waterbath temperature of approximately 60°C.

It is advisable to rinse the walls of the conical flask with 0.5 ml dichloromethane to collect the entire residue in the tip of the flask. The dichloromethane is removed by N-EVAP.

50 µl of acetonitrile are pipetted into the conical flask. Care has to be taken that the entire inner surface is rinsed with the solvent and the dry residue is totally re-dissolved. 0.45 ml of an aqueous solution of hydrochloric acid (SM I) is added and thoroughly mixed with the acetonitrile.

The final solution is transferred into a HPLC vial. For analysis 200 µl are injected into the HPLC.

5.9 Quantification of the Metabolites by Analytical HPLC

Quantification of the metabolites and further separation from interfering matrix compounds is performed by HPLC with column switching on a NH_2-/CN-column combination.

Separation on the NH_2-precolumn is performed by isocratic elution with a solvent mixture which contains acetonitrile + ddH_2O + formic acid in volumes of 10 + 90 + 1.0 (NH_2-column elution mixture SM VIII). Using appropriate equipment (see chapter 4.3) the metabolites are automatically collected from the NH_2-column onto the analytical CN-column during the time windows specified below if the column is eluted at a buffer flow rate of 0.7 ml/minute.

The solvent gradients and the time windows for the collection of metabolite containing fractions are specified below for muscle, fat, liver and kidney. They were used for the in-house validations at BASF but are given as examples only.

The retention time of the analytes may vary with the type of sample material. In addition, as outlined in chapter 4.2.7, the retention times strongly depend on the activity and, to a minor degree, the "age" of the NH_2-column.

Note:
It is essential to test the performance of the NH_2-column in use with the sample material under investigation. Prior to validation experiments and/or routine analyses, the optimal concentration of formic acid in the Amino-HPLC elution buffer (SM VIII) must be adjusted for the NH_2-column in use (see 4.2.7). As is common practise with analytical HLPC analysis, it is advisable to "stabilize" the on-column retention times of the metabolites before injecting the first sample of an analytical series. For this the entire gradient program for both columns should be pre-run using extract from an untreated sample.

In routine analysis with method 354/2 with optimized NH_2-column the run-to-run variation of the metabolite retention times on the analytical HPLC is smaller than 20 seconds.

Analytical chromatography and quantification of all metabolites on the cyano (CN-) column is performed with linear gradients of acetonitrile in acidic ddH_2O [CN-HPLC elution buffers A and B (SM IX and XI)] and a flow rate of 1 ml/minute.

The on-column conditions as well as the NH_2-column switching times are specified below:

On-column conditions for muscle:

The buffer gradients and collection times were applied to the GLP-validation, the results of which are shown in chapter 11.1.1.

Amino-column switching:

The two analytes (BF 490-1 and BF 490-2) are collected onto the analytical HPLC column during a collection "window" from 11.2 to 13.9 minutes.
The entire program for the NH_2-/CN-HPLC buffer gradient is outlined below:

	Entire HPLC-Gradient		Amino-column condition		
Time (minutes)	**Buffer A (SM IX) (Vol. %)**	**Buffer B (SM XI) (Vol. %)**	**Typ of Gradient**	**Amino-col. Buffer (Vol. %)**	**Fraction from NH_2-col. collected to CN -col.**
0	100	0	isocratic	100	
(11.25					I, on
13.90)					I, off
14.05	100	0	start		
37.00	0	100	linear		
39.00	0	100	linear		
39.50	100	0	isocratic		
45.00	100	0	stop		

Analyte retention times:

Applying the chromatography conditions outlined above, typical peak retention times of the analytes after HPLC on a NH_2-/CN-column combination are:

BF 490-2: approximately 24.3 minutes.
BF 490-1: approximately 31 minutes

On-column conditions for kidney and fat:

The buffer gradients and collection times were applied to the GLP-validation, the results of which are shown in chapters 11.1.2 and 11.1.3.

Precolumn with column switching:

The three analytes (BF 490-1, BF 490-2 and BF 490-9) are collected onto the analytical HPLC column during two collection "windows" from 7.9 to 9.5 minutes (BF 490-1, BF 490-2) and 10.8 to 13.4 minutes (BF 490-9).

The entire program for the NH_2-/CN-HPLC buffer gradient is outlined below:

	Entire HPLC-Gradient		Amino-column condition		
Time (min.)	**Buffer A (SM IX) (Vol.%)**	**Buffer B (SM XI) (Vol.%)**	**Typ of Gradient**	**Amino-col. Buffer (Vol.%)**	**Fraction from NH_2-col. collected to CN-col.**
0	100	0	isocratic	100	
7.9					I, on
9.5					I, off
10.8					II, on
13.4					II, off
20.0	100	0	start		
28.0	80	20	linear		
38.0	0	100	linear		
39.0	0	100	isocratic		
39.5	100	0	linear		
45.0	100	0	isocratic		
45.0	100	0	stop		

Analyte retention times:

Applying the chromatography conditions outlined above, typical peak retention times of the analytes after HPLC on a NH_2-/CN-column combination are:

BF 490-2: approximately 26.1 minutes
BF 490-9: approximately 27.1 minutes
BF 490-1: approximately 35.7 minutes

On-column conditions for liver:

The buffer gradients and collection times were applied to the GLP-validation, the results of which are shown in chapter 11.1.4.

Precolumn with column switching:

The analytes (BF 490-1 and BF 490-9) are collected onto the analytical HPLC column during two collection "windows" from 8.5 to 10.05 minutes (BF 490-1) and 11.5 to 13.6 minutes (BF 490-9).

The entire program for the NH_2-/CN-HPLC buffer gradient is outlined below:

	Entire HPLC-Gradient		Amino-column condition		
Time (min.)	**Buffer A (SM IX) (Vol.%)**	**Buffer B (SM XI) (Vol.%)**	**Typ of Gradient**	**Amino-col. Buffer (Vol.%)**	**Fraction from NH_2-col. collected to CN-col.**
0	100	0	isocratic	100	
8.50					I, on
10.05					I, off
11.50					II, on
13.60					II, off
13.80	100	0	Start/linear		
37.00	0	100	isocratisch		
39.00	0	100	linear		
39.50	100	0	isocratic		
45.00	100	0	stop		

Analyte retention times:

Applying the chromatography conditions outlined above, typical peak retention times of the analytes after HPLC on a NH_2- /CN-column combination are:

BF 490-9: approximately 25 minutes
BF 490-1: approximately 31 minutes

Note:
The retention times of the metabolites may vary depending on the equipment and the specifications of the HPLC-columns used.

6. CALCULATION

The individual concentrations of BF 490-1, BF 490-2 and of BF 490-9 in muscle, fat and kidney are calculated as shown in equations I.a-c:

I.a

$$TR_{BF\ 490-1} = \frac{V_{end} \cdot C_{BF\ 490-1}}{V_i \cdot S_m} \cdot A_F$$

I.b

$$TR_{BF\ 490-2} = \frac{V_{end} \cdot C_{BF\ 490-2}}{V_i \cdot S_m} \cdot A_F$$

I.c

$$TR_{BF\ 490-9} = \frac{V_{end} \cdot C_{BF\ 490-9}}{V_i \cdot S_m} \cdot A_F$$

TR	=	Total residues of BF 490-1, BF 490-2 or BF 490-9 in the sample [mg/kg sample material (ppm)]
V_{end}	=	End volume of the extract after all dilution steps [ml]
$C_{BF\ 490-2}$	=	Amount of analyte (e.g. BF 490-2) in the injection volume as read from the calibration curve [ng]
V_i	=	Extract volume injected into the HPLC (aliquot of V_{end}) [µl]
S_m	=	Weight of the sample extracted [g]
A_F	=	Aliquot of the total extract which is used in V_{end}.

If residue data are to be corrected for loss of analyte during sample extraction and clean-up procedures the total residues [TR] have to be corrected with the results of the procedural recoveries as shown in equation II:

II.

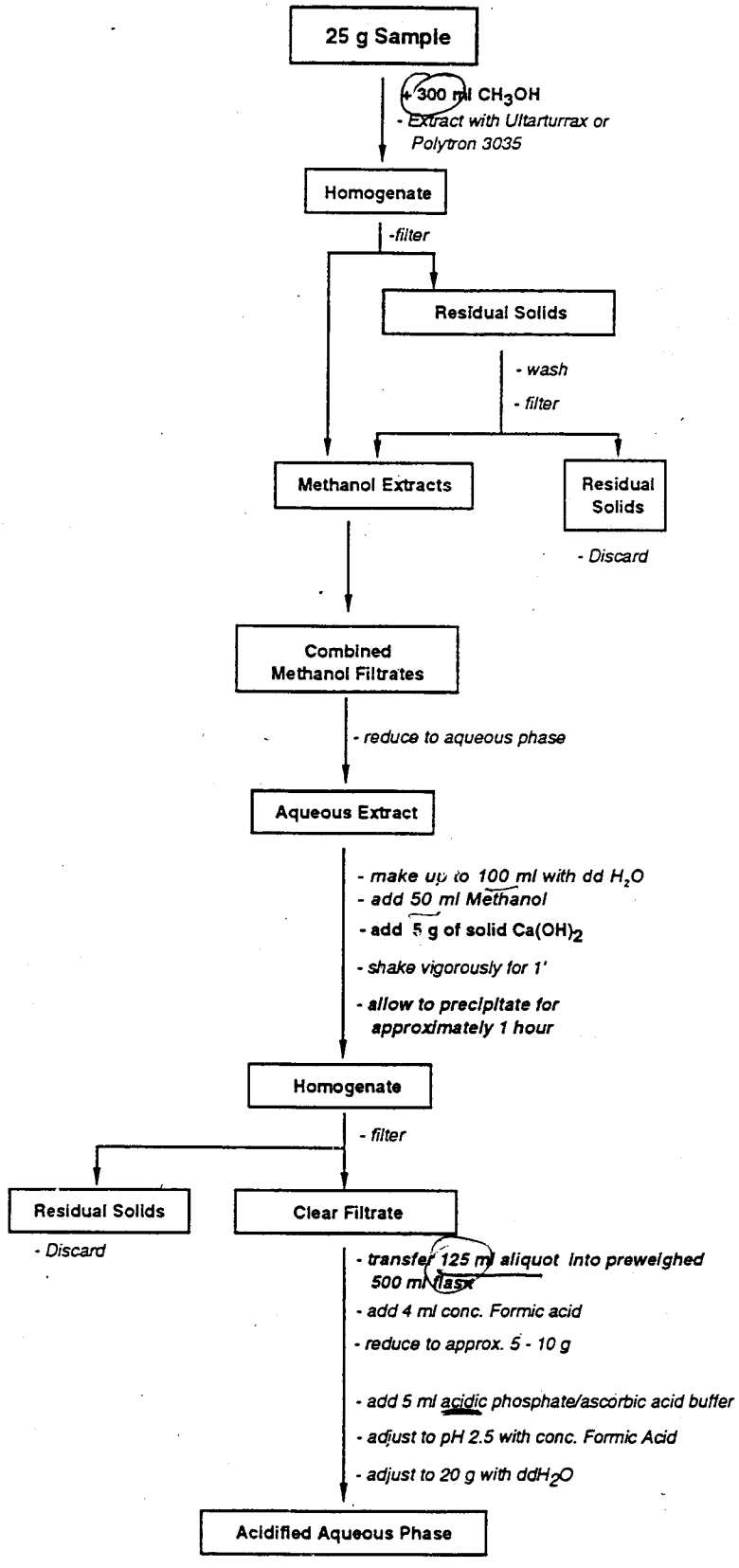
$$TR_{RC} = TR \cdot R_{FE}$$

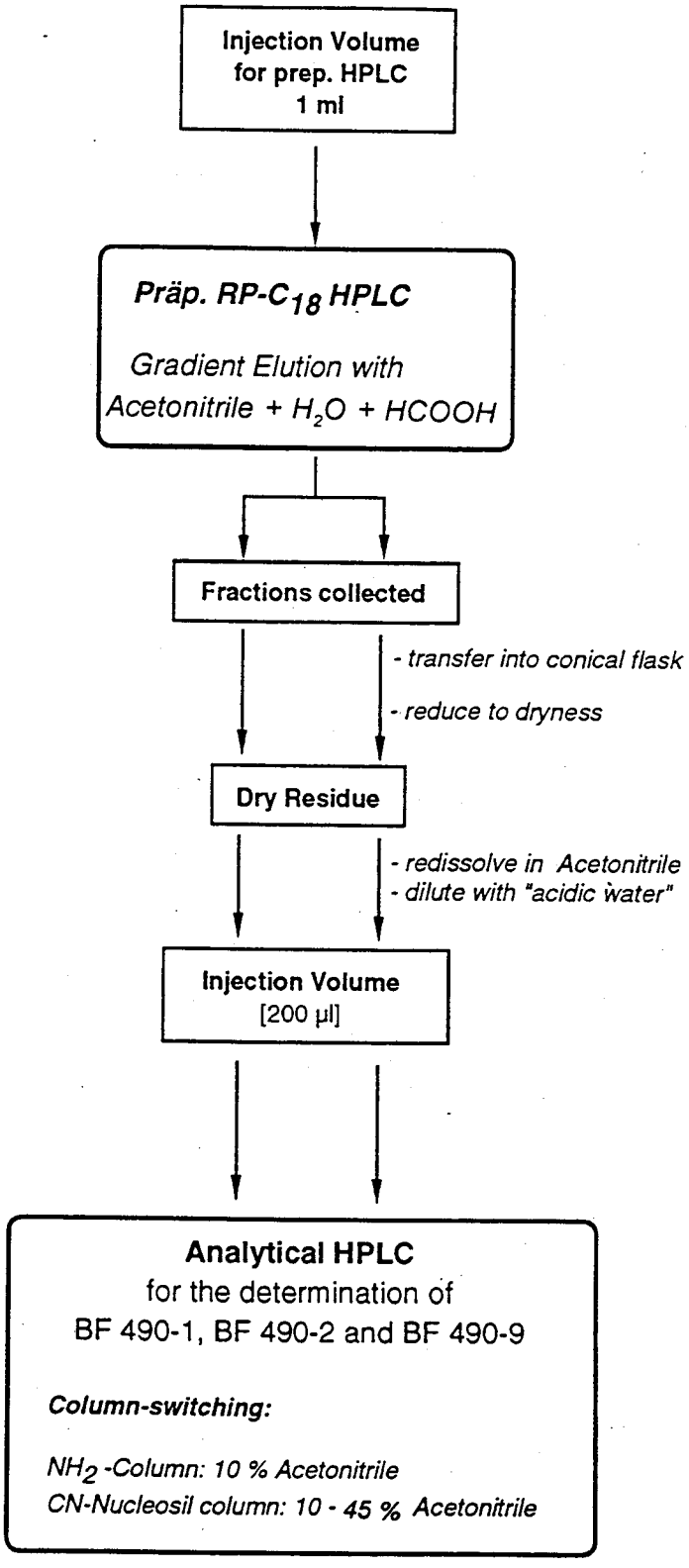
TR_{RC}	=	Residue concentration of the analyte in the sample corrected with the procedural recovery of the analyte in fortification experiments [mg/1000 g sample material (ppm)]
TR_{FE}	=	Procedural recovery of the analyte as determined from fortification experiments performed in parallel to the sample analysis (see 5.1)

$$TR_{FE} = \frac{100\%\ \text{(level of fortification)}}{\%\ \text{Recovery}}$$

For routine analysis requirements residue data should not be corrected for procedural recoveries. Results of fortification experiments should be listed individually.

7. FLOW DIAGRAM OF METHOD 354/2




Injection Volume
for prep. HPLC
1 ml
Präp. RP-C18 HPLC
Gradient Elution with
Acetonitrile + H2O + HCOOH
Fractions collected
- transfer into conical flask
- reduce to dryness
Dry Residue
- redissolve in Acetonitrile
- dilute with "acidic water"
Injection Volume
[200 µl]
Analytical HPLC
for the determination of
BF 490-1, BF 490-2 and BF 490-9
Column-switching:
NH2 -Column: 10 % Acetonitrile
CN-Nucleosil column: 10 - 45 % Acetonitrile

8. METHOD MANAGEMENT AND TIME REQUIREMENT

The analysis of one series of samples (= 7 unknown samples, 2 fortified samples for recovery experiments, 1 blank sample) requires two and a half working days (20 hours) per laboratory assistant. This time includes the calculation of the results, the preparation of the equipment as well as the reporting of all raw data under GLP.

9. POTENTIAL PROBLEMS

In case of an high content of fat in liver and kidney samples, a further extract clean-up step may be included in the technical procedure (see chapter 5.) to eliminate possible interferences of the analyte signals with non-polar matrix compounds du HPLC analysis.

For this a 125 ml aliquot of the filtrate from 5.3 is transferred into a preweighed 500 ml round bottom flask. The methanol is removed from the filtrate by rotary evaporation at about 250 mbar and a water bath temperature of approximately 60°C until the extract is concentrated to about 10 to 15 g (aqueous phase). To this aqueous extract 5 ml of the alkaline phosphate/ascorbic acid buffer (SM X) are added. The extract is filled up to approximately 60 g with ddH_2O after which the pH of the solution is adjusted to 9.5 with NaOH solution (1 M). The alkaline extract is subsequently adjusted to a total mass of exactly 85 g with ddH_2O. Finally 15 ml of methanol are added. The extract is transferred into a separatory funnel (250 ml). The round bottom flask is rinsed with two volumes of 50 ml each of the ethylacetate/MTBE-mixture (SM III) which are transferred into the separatory funnel, too. The aqueous phase is extracted to remove fat and fatty acids. The aqueous layer is transferred back into the same one liter round bottom flask, the organic phase is discarded. The aqueous phase is transferred back into the separatory funnel and extracted once more against 100 ml of the ethylacetate/MTBE-mixture in a similar procedure as described above. The aqueous layer is again transferred back the same one liter round bottom flask, the organic phase is discarded. After the addition of 3.5 ml concentrated formic acid the aqueous phase is concentrated to a total weight of approximately 10 - 15 g using a rotary evaporator at 50 mbar reduced pressure and a water bath temperature of approximately 60°C.

The pH of the acidified, concentrated extract is adjusted to 2.5 with concentrated formic acid (approximately 1 ml). The total weight of the extract is adjusted to 20 g with ddH_2O. This extract is tranferred into a 100 ml separatory funnel and phase partitioned against DCM as outlined in chapter 5.4.

To maintain the level of reliability shown for this method it is important to ensure that all steps within the method protocol, especially the transfer of extracts from large to small volume containers are performed with outmost care and exactly as specified in the protocol.

10. CONFIRMATORY TECHNIQUES

As alternative for the analytical HPLC with UV-detection a LC/MS-system may be used as confirmatory technique (tested under non GLP conditions).

The analytical procedure and the preparation of the injection volume should be followed as described in the chapters 5.1 to 5.8. For the analytical HPLC only the NH_2-column with the specifications described in chapter 4.3.2. is required. The analytical conditions as outlined below allow the quantification of all three analytes at a concentration level of 0.01 mg/kg each.

HPLC/MS-conditions:

Column:	NH_2-column as specified in chapter 4.3.2 (Precolumn specification)
Mode of elution:	Isocratic, with 10 % acetonitrile and 1.0 % formic acid at a pH of about 1.0 % (Precolumn elution buffer (XIII))
Flow rate:	0.7 ml/min
Injection volume:	50 µl
Split:	95/5
Ionization:	Ion spray
Detection:	Mass 333
Instruments used:	for example: Waters 600-MS (HPLC) and PE-SCIEX API III SN014 (MS)

11. RECOVERIES

The recovery data listed in the chapter 11.1 and the tables 1 to 6 were determined during GLP and non-GLP validation studies of method 354/2 which were conducted in-house at the BASF testing facility.

Simultaneously BASF method 354/2 was transferred to and validated under GLP in an external laboratory in the United Kingdom. These data are cited and summarized in chapter 11.2 and the tables 7 to 10.

11.1 In-house Validation Data

Fortified sample recovery efficiency values are expressed as percentage (%). The recovery data are corrected for interferences of matrix compounds of the appropriate unfortified sample. If not stated otherwise, apparent analyte concentrations in the unfortified samples were below the limit of detection of 2 ppb. Non-GLP validations were performed unter GLP-equivalent conditions. However, the data were not audited by QAU. The aim of these validations was to test the ruggedness and repeatability of the method.

11.1.1 Validation in Muscle

Table 1: Recoveries of BF 490-1 and BF 490-2 in muscle

Date of analysis: 22.06.1994
GLP-Compliance: No

Analyte	Level of fortification [mg/kg]	Individual Recoveries [%]	Mean Recovery [%]	Std. Dev. [+/-%]	Coeff. Var.. [+/-%]
BF 490-1	0.01	117.1 79.9 77.5 81.1	88.9	18.9	21.2
	1.00	82.7 82.3 86.8 82.7	83.6	2.1	2.5
		Total:	86.3	12.7	14.8
BF 490-2	0.01	85.9 83.9 85.8 98.1	88.4	6.5	7.4
	1.00	86.0 87.2 91.0 90.6	88.7	2.5	2.8
		Total:	88.6	4.6	5.2

Table 2: Recoveries of BF 490-1 and BF 490-2 in muscle

Date of analysis: 26.07.1994
GLP-Compliance: Yes
Queue-File: ZS 94006

Analyte	Level of fortification [mg/kg]	Individual Recoveries [%]	Mean Recovery [%]	Std. Dev. [+/-%]	Coeff. Var. [+/-%]
BF 490-1	0.01	79.5 84.2 76.6 79.3	79.9	3.2	4.0
	1.00	85.2 86.3 86.2 86.1	86.0	0.5	0.6
		Total:	82.9	3.9	4.6
BF 490-2	0.01	90.1 86.7 89.4 81.3	86.9	4.0	4.6
	1.00	84.8 84.5 85.8 88.0	85.8	1.6	1.8
		Total:	86.3	2.9	3.3

11.1.2 Validation in Fat

Table 3: Recoveries of BF 490-1, BF 490-2 and BF 490-9 in fat

Date of analysis: 01.09.1994
GLP-Compliance: Yes
Queue-File: WD 94033

Analyte	Level of fortification [mg/kg]	Individual Recoveries [%]	Mean Recovery [%]	Std. Dev. [+/-%]	Coeff. Var. [+/-%]
BF 490-1	0.01	48.9 65.7 56.8 94.4	66.5	19.9	29.9
	1.00	69.6 71.7 70.8 71.8	71.0	1.0	1.4
		Total:	68.7	13.2	19.3
BF 490-2	0.01	83.9 96.9 92.1 102.9	94.0	8.0	8.5
	1.00	83.8 83.0 82.2 83.2	83.1	0.7	0.8
		Total:	88.5	7.9	8.9
BF 490-9	0.01	81.7 101.7 101.5 88.4	93.3	9.9	10.7
	1.00	75.6 74.3 74.1 74.4	74.6	0.7	0.9
		Total:	84.0	11.9	14.2

11.1.3 Validation in Kidney

Table 4: Recoveries of BF 490-1, BF 490-2 and BF 490-9 in kidney

Date of analysis: 22.06.1994
GLP-Compliance: No

Analyte	Level of fortification [mg/kg]	Individual Recoveries [%]	Mean Recovery [%]	Std. Dev. [+/-%]	Coeff. Var. [+/-%]
BF 490-1	0.01	82.6 93.1 91.9 121.8	97.4	17.0	17.4
	1.00	79.3 76.4 86.5 75.8	79.5	4.9	6.2
		Total:	88.4	15.0	17.0
BF 490-2	0.01	92.7 98.3 84.7 88.2	91.0	5.9	6.5
	1.00	82.8 75.0 82.3 81.4	80.4	3.6	4.5
		Total:	85.7	7.3	8.5
BF 490-9	0.01	112.4 111.0 103.9 110.2	109.4	3.8	3.4
	1.00	83.5 77.7 81.9 82.2	81.3	2.5	3.1
		Total:	95.4	15.3	16.0

11.1.4 Validation in Liver

Table 5: Recoveries of BF 490-1 and BF 490-2 in liver

Date of analysis: 28.06.1994
GLP-Compliance: No

Analyte	Level of fortification [mg/kg]	Individual Recoveries [%]	Mean Recovery [%]	Std. Dev. [+/-%]	Coeff. Var. [+/-%]
BF 490-1	0.01	72.4 88.4 81.9 73.9	79.2	7.4	9.4
	1.00	81.0 80.1 80.5 78.4	80.0	1.1	1.4
		Total:	79.6	4.9	6.2
BF 490-2	0.01	90.5 97.7 88.7 93.4	92.6	3.9	4.2
	1.00	90.2 95.5 91.3 95.3	93.1	2.7	2.9
		Total:	92.8	3.1	3.4

Table 6: Recoveries of BF 490-1 and BF 490-9 in liver

Date of analysis: 04.07.1994
GLP-Compliance: No

Analyte	Level of fortification [mg/kg]	Individual Recoveries [%]	Mean Recovery [%]	Std. Dev. [+/-%]	Coeff. Var. [+/-%]
BF 490-1	0.01	79.3 104.2 72.3 79.2	83.8	14.0	16.7
	1.00	75.5 78.7 77.2 75.0	76.6	1.7	2.2
		Total:	80.2	10.0	12.5
BF 490-9	0.01	102.9 101.2 102.9 101.9	102.1	0.7	0.7
	1.00	76.6 71.3 71.1 67.0	71.5	3.9	5.5
		Total:	86.8	16.6	19.1

11.2 External Validation Data

The data listed below are taken from external GLP validation studies compiled in the final report of HRC study protocol BSF 528 (Ref. 1)

Fortified sample recovery efficiency values are expressed as percentage (%). The recovery data are corrected for interferences of matrix compounds of the appropriate unfortified sample. If not stated otherwise, apparent analyte concentrations in the unfortified samples were below the limit of detection of 2 ppb.

11.2.1 Validation in Muscle

Table 7: Recoveries of BF 490-1 and BF 490-2 in muscle

Analyte	Level of fortification [mg/kg]	Individual Recoveries [%]	Mean Recovery [%]	Std. Dev. [+/-%]	Coeff. Var. [+/-%]
BF 490-1	0.01	97.7 96.5 90.0	94.7	4.1	4.4
	1.00	82.7 92.2 86.9 90.3	88.0	4.2	4.7
		Total:	90.9	5.2	5.7
BF 490-2	0.01	86.6 106.0 94.5	95.7	9.8	10.2
	1.00	68.8 89.1 79.9 85.8	80.9	8.9	11.0
		Total:	87.2	11.6	13.3

11.2.2 Validation in Fat

Table 8: Recoveries of BF 490-1, BF 490-2 and BF 490-9 in fat

Analyte	Level of fortification [mg/kg]	Individual Recoveries [%]	Mean Recovery [%]	Std. Dev. [+/-%]	Coeff. Var. [+/-%]
BF 490-1	0.01	89.3 90.4 74.2 61.3 74.4 87.8 92.7 80.0	81.3	10.8	13.3
	1.00	82.5 53.0 50.7 67.5	63.4	14.7	23.2
		Total:	75.3	14.5	19.3
BF 490-2	0.01	84.7 65.8 92.6 111.0 120.0 86.7 63.7 87.3	89.0	19.5	21.9
	1.00	66.0 54.9 68.1 75.2	66.1	8.4	12.7
		Total:	81.3	19.7	24.2
BF 490-9	0.01	98.9 93.8 113.0 106.0 112.0 152.0 85.9 108.0	108.7	19.8	18.2
	1.00	65.5 49.6 85.1 85.1	71.3	17.2	24.1
		Total:	96.2	25.9	26.9

11.2.3 Validation in Kidney

Table 9: Recoveries of BF 490-1, BF 490-2 and BF 490-9 in kidney

Analyte	Level of fortification [mg/kg]	Individual Recoveries [%]		Mean Recovery [%]	Std. Dev. [+/-%]	Coeff. Var. [+/-%]
BF 490-1	0.01	80.7 68.6	93.8	81.0	12.6	15.6
	1.00	84.2 77.5	85.4	82.4	4.3	5.2
			Total:	81.7	8.5	10.3
BF 490-2	0.01	77.8 69.0	82.1	76.3	6.7	8.8
	1.00	93.8 85.0	95.7	91.5	5.7	6.2
			Total:	83.9	10.0	14.2
BF 490-9	0.01	95.1 78.4	81.6	85.0	8.9	10.4
	1.00	90.7 88.5	66.0	81.7	13.7	16.7
			Total:	83.4	10.5	12.5

11.2.4 Validation in Liver

Table 10: Recoveries of BF 490-1 and BF 490-9 in liver

Analyte	Level of fortification [mg/kg]	Individual Recoveries [%]		Mean Recovery [%]	Std. Dev. [+/-%]	Coeff. Var. [+/-%]
BF 490-1	0.01	74.8 73.0	76.7	74.8	1.9	2.5
	1.00	80.2 71.1	69.4	73.6	5.8	7.9
			Total:	74.2	3.9	5.3
BF 490-9	0.01	84.0 87.0	76.7	82.6	5.3	6.4
	1.00	109.0 105.0	77.2	97.1	17.3	17.8.
			Total:	89.8	13.9	15.5

11.3.1 Repeatability

The results of repeatability calculations are compiled in the tables 11 to 14 for each analyte, tissue and level of fortification. A summary of the reapeatability data is shown in table 20. The recovery data were taken from the tables 1 to 10 and are listed for each individual validation study. External validation studies were performed at HRC in the United Kindgom (see Ref. 1).

Individual validation studies in each laboratory were performed in identical test material: untreated sample material fortified on one level of concentration of all analytes was taken from one sample container. All samples in a validation study were fortified from an identical spiking solution and were simultaneously processed by one laboratory assistant. For the results of differences in tables 11 to 14 only the absolute differences are shown, probability levels of 90 % were not calculated.

Table 11: Repeatability of method 354/2 in muscle

Level of fortification [mg/kg]	Analyte	Range of Recoveries [%]	Difference [%]	Type of Validation
0.01	BF 490-1	77.5 to 117.1	39.6	non GLP
		76.6 to 84.2	7.6	GLP
		90.0 to 97.7	7.7	External
	BF 490-2	83.9 to 98.1	14.2	non GLP
		81.3 to 90.1	8.8	GLP
		86.6 to 106.0	19.4	External
1.00	BF 490-1	82.3 to 86.8	4.5	non GLP
		85.2 to 86.3	1.1	GLP
		82.7 to 92.2	9.5	External
	BF 490-2	86.0 to 91.0	5.0	non GLP
		84.5 to 88.0	3.5	GLP
		68.8 to 89.1	20.3	External

Table 12: Repeatabilitv of method 354/2 in fat

Level of fortification [mg/kg]	Analyte	Range of Recoveries [%]	Difference [%]	Type of Validation
0.01	BF 490-1	48.9 to 94.4	45.5	GLP
		61.3 to 92.7	31.4	External
	BF 490-2	83.9 to 102.9	19.0	GLP
		63.7 to 120.0	56.3	External
	BF 490-9	81.7 to 101.7	20.0	GLP
		85.9 to 152.0	66.1	External
1.00	BF 490-1	69.6 to 71.8	2.2	GLP
		50.7 to 82.5	31.8	External
	BF 490-2	82.2 tot 83.8	1.6	GLP
		54.9 o 75.2	20.3	External
	BF 490-9	74.1 to 75.6	1.5	GLP
		49.6 to 85.1	35.5	External

Table 13: Repeatability of method 354/2 in kidney

Level of fortification [mg/kg]	Analyte	Range of Recoveries [%]	Difference [%]	Type of Validation
0.01	BF 490-1	82.6 to 121.8	39.2	non-GLP
		68.6 to 93.8	25.2	External
	BF 490-2	84.7 to 98.3	13.6	non-GLP
		69.0 to 82.1	13.1	External
	BF 490-9	103.9 to 112.4	8.5	non-GLP
		78.4 to 95.1	16.7	External
1.00	BF 490-1	75.8 to 86.5	10.7	non-GLP
		77.5 to 85.4	7.9	External
	BF 490-2	75.0 tot 82.8	7.8	non-GLP
		85.0 o 95.7	10.7	External
	BF 490-9	77.7 to 83.5	5.8	non-GLP
		66.0 to 90.7	24.7	External

Table 14: Repeatability of method 354/2 in liver

Level of fortification [mg/kg]	Analyte	Range of Recoveries [%]	Difference [%]	Type of Validation
0.01	BF 490-1	72.4 to 88.4	16.0	non GLP
		72.3 to 104.2	31.9	non-GLP
		73.0 to 76.7	3.7	External
	BF 490-2	88.7 to 97.7	9.0	non GLP
	BF-490-9	101.2 to 102.9	1.7	non-GLP
		76.7 to 87.0	10.3	External
1.00	BF 490-1	78.4 to 81.0	2.6	non GLP
		75.0 to 78.7	3.7	non-GLP
		69.4 to 80.2	10.8	External
	BF 490-2	90.2 to 95.5	5.3	non GLP
	BF 490-9	67.0 to 76.6	9.6	non-GLP
		77.2 to 109.0	31.8	External

11.3.2 Reproducibility

The results of reproducibility studies are compiled in the tables 15 to 18 for each analyte, tissue, level of fortification and performing laboratory. A summary of the reproducibility data is shown in table 21. External validation studies were performed at HRC in the United Kingdom (see Ref. 1). Internal and external validation studies were different with respect to instrumentation and time. They were performed by different laboratory personnel.

Individual validation studies in each laboratory were performed in identical test material: untreated sample material fortified on one level of concentration of all analytes was taken from one sample container. All samples in a validation study were fortified from an identical spiking solution and were simultaneously processed by one laboratory assistant. For the results of differences in tables 15 to 18 only the absolute differences are shown, probability levels of 90 % were not calculated.

Table 15: Reproducibility of method 354/2 in muscle

Fort. Level [mg/kg]	Analyte	Site of Validation	Recoveries Range [%]	Difference [%]	Mean [%]	Reproducibility [%]
0.01	BF 490-1	in-house external	76.6 to 84.2 90.0 to 97.7	7.6 7.7	79.9 94.7	
Comparison int. and ext. lab.:			76.6 to 97.7	21.1		14.8
0.01	BF 490-2	in-house external	81.3 to 90.1 86.6 to 106.0	8.8 19.4	86.9 95.7	
Comparison int. and ext. lab.:			81.3 to 106.0	24.7		8.8
1.00	BF 490-1	in-house external	85.2 to 86.3 82.7 to 92.2	1.1 9.5	86.0 88.0	
Comparison int. and ext. lab.:			82.7 to 92.2	9.5		2.0
1.00	BF 490-2	in-house external	84.5 to 88.0 68.8 to 89.1	3.5 20.3	85.8 80.9	
Comparison int. and ext. lab.:			68.8 to 89.1	20.3		4.9

For in-house data the GLP-data from table 11 were used.

Table 16: Reproducibility of method 354/2 in fat

Fort. Level [mg/kg]	Analyte	Site of Validation	Recoveries Range [%]	Difference [%]	Mean [%]	Reproducibility [%]
0.01	BF 490-1	in-house external	48.9 to 94.4 61.3 to 92.7	45.5 31.4	66.5 81.3	
Comparison int. and ext. lab.:			48.9 to 94.4	45.5		14.8
0.01	BF 490-2	in-house external	83.9 to 102.9 63.7 to 120.0	19.0 56.3	94.0 89.0	
Comparison int. and ext. lab.:			63.7 to 120.0	56.3		5.0
0.01	BF 490-9	in-house external	81.7 to 101.7 85.9 to 152.0	20.0 66.1	93.3 108.7	
Comparison int. and ext. lab.:			81.7 to 152.0	70.3		15.4
1.00	BF 490-1	in-house external	69.6 to 71.8 50.7 to 82.5	2.2 31.8	71.0 63.4	
Comparison int. and ext. lab.:			50.7 to 82.5	31.8		7.6
1.00	BF 490-2	in-house external	82.2 to 83.8 54.9 to 75.2	1.6 20.3	83.1 66.1	
Comparison int. and ext. lab.:			54.9 to 83.8	28.9		17.0
1.00	BF 490-9	in-house external	74.1 to 75.6 49.6 to 85.1	1.5 35.5	74.6 71.3	
Comparison int. and ext. lab.:			49.6 to 85.1	35.5		3.3

Table 17: Reproducibility of method 354/2 in kidney

Fort. Level [mg/kg]	Analyte	Site of Validation	Recoveries Range [%]	Difference [%]	Mean [%]	Reproducibility [%]
0.01	BF 490-1	in-house external	82.6 to 121.8 68.6 to 93.8	39.2 25.2	97.4 81.0	
Comparison int. and ext. lab.:			68.6 to 121.8	53.2		16.4
0.01	BF 490-2	in-house external	84.7 to 98.3 69.0 to 82.1	13.6 13.1	91.0 76.3	
Comparison int. and ext. lab.:			69.0 to 98.3	29.3		14.7
0.01	BF 490-9	in-house external	103.9 to 112.4 78.4 to 95.1	8.5 16.7	109.4 85.0	
Comparison int. and ext. lab.:			78.4 to 112.4	34.0		24.4
1.00	BF 490-1	in-house external	75.8 to 86.5 77.5 to 85.4	10.7 7.9	79.5 82.4	
Comparison int. and ext. lab.:			75.8 to 86.5	10.7		2.9
1.00	BF 490-2	in-house external	75.0 to 82.8 85.0 to 95.7	7.8 10.7	80.4 91.5	
Comparison int. and ext. lab.:			75.0 to 95.7	20.7		11.1
1.00	BF 490-9	in-house external	77.7 to 83.5 66.0 to 90.7	5.8 24.7	81.3 81.7	
Comparison int. and ext. lab.:			66.0 to 90.7	24.7		0.4

The in-house data were not generated under GLP.

Table 18: Reproducibility of method 354/2 in liver

Fort. Level [mg/kg]	Analyte	Site of Validation	Recoveries Range [%]	Difference [%]	Mean [%]	Reproducibility [%]
0.01	BF 490-1	in-house external	72.3 to 104.2 73.0 to 76.7	31.9 3.7	83.8 74.8	
Comparison int. and ext. lab.:			72.3 to 104.2	31.9		9.0
0.01	BF 490-9	in-house external	101.2 to 102.9 76.7 to 87.0	1.7 10.3	102.1 82.6	
Comparison int. and ext. lab.:			76.6 to 102.9	26.3		19.5
1.00	BF 490-1	in-house external	75.0 to 78.7 69.4 to 80.2	3.7 10.8	76.6 73.6	
Comparison int. and ext. lab.:			69.4 to 80.2	10.8		3.0
1.00	BF 490-9	in-house external	67.0 to 76.6 77.2 to 109.0	9.6 31.8	71.5 97.1	
Comparison int. and ext. lab.:			67.0 to 109.0	42.0		25.6

The in-house data were not generated under GLP.

11.4 Limit of Detection

From in-house and external validation studies it can be concluded that the limit of detection for all analytes in the tissues tested is approximately 2 ppb (0.002 mg/kg).

12. SUMMARY OF METHOD AND FINDINGS

12.1 Method

Type of method:	Individual residue method
Test systems:	Skeletal muscle, peritoneal fat (GLP) liver and kidney of beef (non GLP)
Analytes detected:	BF 490-1, BF 490-2 and BF 490-9
Extraction:	25 g sample aliquot in 300 ml methanol
Clean-up:	$Ca(OH)_2$-precipitation, phase partitioning into DCM, SPE NH_2-column, preparative HPLC on C_{18}-column
Determined as:	Intakt, individual analytes
Method of determination:	Analytical HPLC with column switching on a NH_2-/C_{18}-column combination. UV-detection at 270 nm
Confirmatory technique:	LC/MS on analytical HPLC with NH_2-column. Ion spray, detection on mass 333
Time required:	A set of 10 samples requires 20 hours of work

12.2 Findings

Limit of detection: Approximately 0.002 mg/kg for all analytes in all tissues
Limit of quantitation: 0.01 mg/kg for all analytes in all tissues
Specificity: BF 490-1, BF 490-2 and BF 490-9 can be indidividually determined
Levels of fortification: 0.01 and 1.00 mg/kg
Recoveries: A summary of the recoveries is shown in table 19. Data printed in italic are derived from non-GLP studies (see tables 1 to 6)

Table 19: Summary of the in-house recoveries of method 354/2 in beef tissues

Tissue	Analytes	Fort. Level [mg/kg]	Mean Recoveries [%]			Total mean [%]	Mean Std. Dev. [%]			Total mean [%]
Muscle	BF 490-1 BF 490-2	**0.01**	*88.9* 79.9	*88.4* 86.9		***86.0***	*18.9* 3.2	*6.5* 4.0		***8.2***
		1.00	*83.6* 86.0	*88.7* 85.8		***86.0***	*2.1* 0.5	*2.5* 1.6		***1.7***
Liver	BF 490-1 BF 490-2 BF 490-9	**0.01**	*79.2* *83.8*	*92.6* *102.1*		***89.4***	*7.4* *14.0*	*3.9* *0.7*		***6.5***
		1.00	*80.0* *76.6*	*93.1* *71.5*		***80.3***	*1.1* *1.7*	*2.7* *3.9*		***2.4***
Fat	BF 490-1 BF 490-2 BF 490-9	**0.01**	66.5	94.0	93.3	**84.6**	19.9	8.0	9.9	**12.6**
		1.00	71.0	83.1	74.6	**76.2**	1.0	0.7	0.7	**0.8**
Kidney	BF 490-1 BF 490-2 BF 490-9	**0.01**	*97.4*	*91.0*	*109.4*	***99.3***	*17.0*	*5.9*	*3.8*	***8.9***
		1.00	*79.5*	*80.4*	*81.3*	***80.4***	*4.9*	*3.6*	*2.5*	***3.7***
All	All	**0.01** **1.00**				***89.8*** ***80.7***				***9.0*** ***2.1***

Standard deviation: See table 19
Blank values: Must be determined by analyses of control samples. No blank values or < 10 % of signal at 0.01 mg/kg fortification level.
Repeatability: A summary of the repeatability is shown in table 20. Data printed in italic were not produced under GLP-compliance. Underlined data were generated in the external laboratory (see Ref. 1).

Table 20: Summary of the repeatability of method 354/2 in beef tissues

Tissue	Analytes	Fort. Level [mg/kg]	Recovery Differences in Val. Studies [%]			Mean [%]	Repeatability [mg/kg]
Muscle	BF 490-1 BF 490-2	**0.01**	7.7 *14.2*	7.6 8.8	*39.6* 19.4	**16.2**	**0.002**
		1.00	*4.5* *3.5*	9.5 5.0	1.1 20.3	**7.3**	**0.073**
Liver	BF 490-1 BF 490-2 BF 490-9	**0.01**	*16.0* *31.9*	*3.7* *9.0*	*1.7* 10.3	***12.1***	**0.001**
		1.00	*2.6* *3.7*	*10.8* *5.3*	*9.6* 31.8	***10.6***	**0.106**
Fat	*BF 490-1* *BF 490-2* *BF 490-9*	***0.01***	45.5 31.4	19.0 56.3	20.0 66.1	**39.7**	**0.004**
		1.00	2.2 31.8	1.6 20.3	1.5 35.5	**15.5**	**0.155**
Kidney	BF 490-1 BF 490-2 BF 490-9	**0.01**	*39.2* 25.2	*13.6* 13.1	*8.5* 16.7	**19.4**	**0.002**
		1.00	*10.7* 7.9	*7.8* 10.7	*5.8* 24.7	**11.3**	**0.113**
All	All	**0.01** **1.00**				**21.9** **11.2**	**0.003** **0.112**

Reproducibility: A summary of the reproducibility is shown in table 21. Data printed in italic are partly based on non-GLP data (see tables 15 -18).

Table 21: Summary of the reproducibility of method 354/2 in beef tissues

Tissue	Analytes	Fort. Level [mg/kg]	Recovery Differences Int. Lab vs. Ext. Lab [%]			Mean [%]	Reproducibility [mg/kg]
Muscle	BF 490-1 BF 490-2	**0.01**	14.8	8.8		**11.8**	**0.001**
		1.00	2.0	4.9		**3,5**	**0.035**
Liver	BF 490-1 BF 490-9	**0.01**	*9.0*	*19.5*		***14.3***	**0.001**
		1.00	*3.0*	*25.6*		***14.3***	**0.143**
Fat	BF 490-1 BF 490-2 BF 490-9	**0.01**	*14.8*	*5.0*	*15.4*	***11.7***	***0.001***
		1.00	*7.6*	*17.0*	*3.3*	***9.3***	***0.093***
Kidney	BF 490-1 BF 490-2 BF 490-9	**0.01**	*16.4*	*14.7*	*24.4*	***18.5***	***0.002***
		1.00	*2.9*	*11.1*	*0.4*	***4.8***	***0.048***
All	All	**0.01** **1.00**				***14.1*** ***8.0***	***0.001*** ***0.080***

Difficulties encountered: None

13. REFERENCES

1. J.G. Maxwell (1994): Validation of the methods of analysis for BAS 490 F Metabolites in Milk and Tissue from Dairy Cows; BASF Method No. 354/1 (Milk), BASF Method No. 354/2 (Tissue). HRC Study No. BSF 528. Huntingdon Research Centre LTD, Huntingdon, United Kingdom.

2. Mackenroth Ch. and A. Krotzky (1994): Stability of BAS 490 F in Acetone and BF 490-1 in Acetonitrile. BASF Study Code SST-490/1-92. Reg. Doc # BASF 94/11098. BASF Aktiengesellschaft, Limburgerhof, Germany.

3. Mackenroth Ch. and A. Krotzky (1994): Determination of the Stability of BF 490-2, BF 490-6, BF 490-9 and BF 490-18 in Acetonitrile. BASF Study Code SST/001/94. Reg.Doc # BASF 94/11106. BASF Aktiengesellschaft, Limburgerhof, Germany.

4. Mackenroth Ch. and A. Krotzky (1994): Method for the Determination of BF 490-1, BF 490-2, and BF 490-9 and BF 490-18 in Muscle, Liver, Fat and Kidney of Beef; Technical Procedure from 29.09.1994. BASF-Report No. 3991. BASF Aktiengesellschaft, Limburgerhof, Germany.

5. Mayer F. (1994): The Metabolism of ^{14}C-BAS 490 F in the Goat; Reg. Doc. # BASF 94/11104. BASF Aktiengesellschaft, Limburgerhof, Germany.

14. TYPICAL HPLC ELUTION PROFILES

Figure 1: Standard Chromatogram containing 0.25 µg/ml BF 490-1 and BF 490-2 each

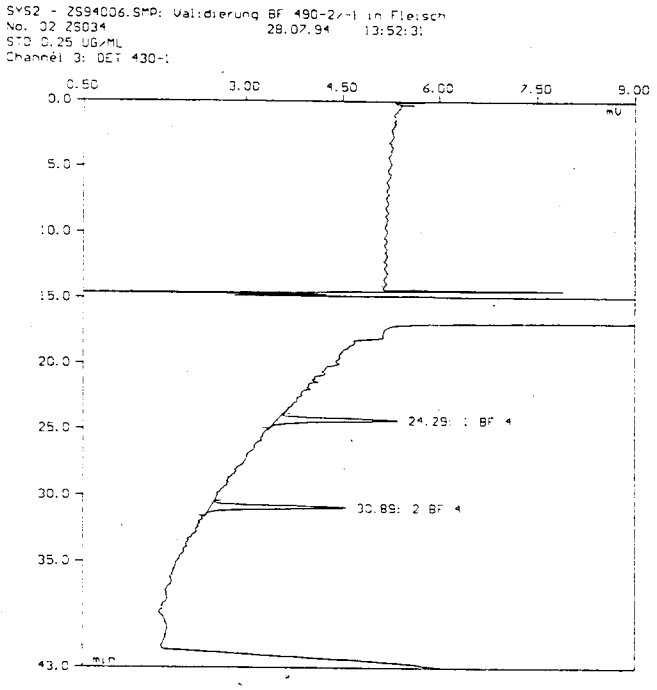


Figure 2: HPLC elution profile of an untreated muscle sample

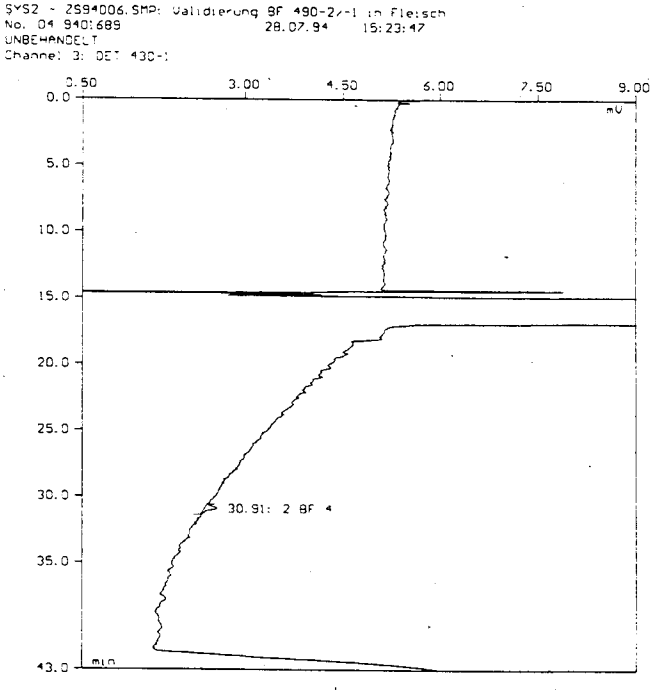


Figure 3: HPLC elution profile of an untreated muscle sample fortified with BF 490-1 and BF 490-2 at a level of 0.010 mg/kg each

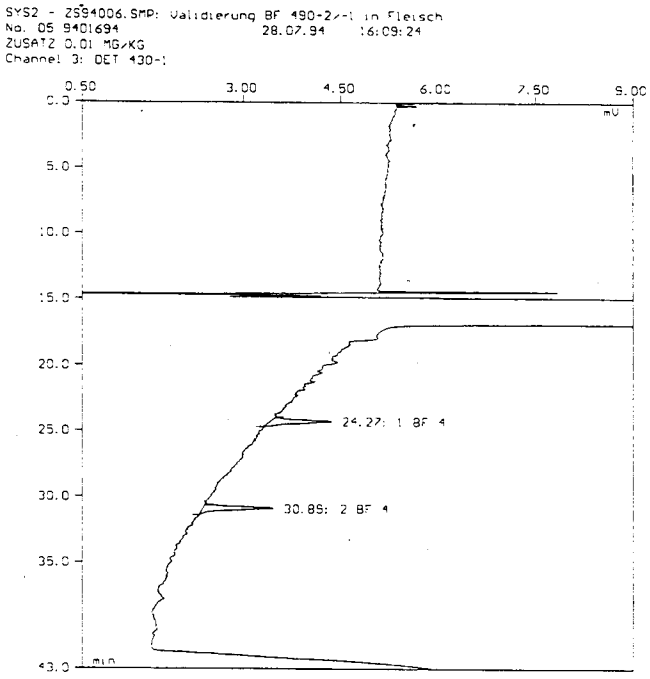


Figure 4: HPLC elution profile of an untreated muscle sample fortified with BF 490-1 and BF 490-2 at a level of 1.00 mg/kg each

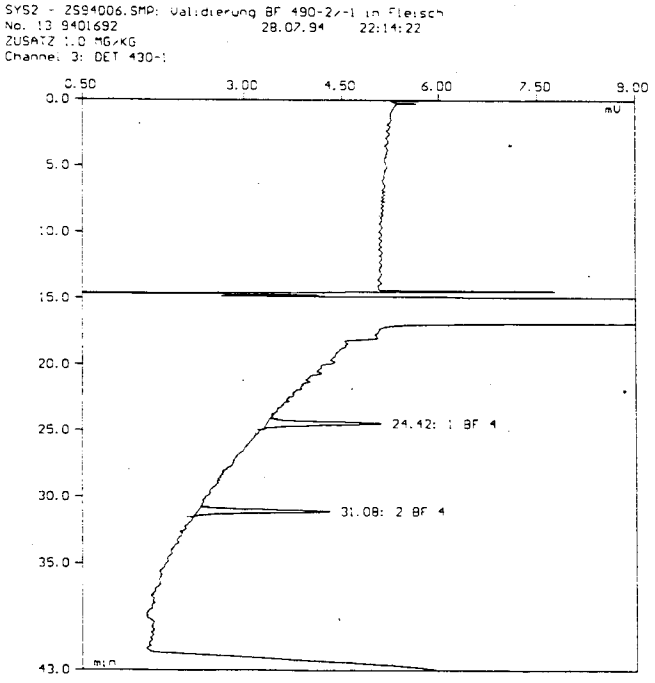
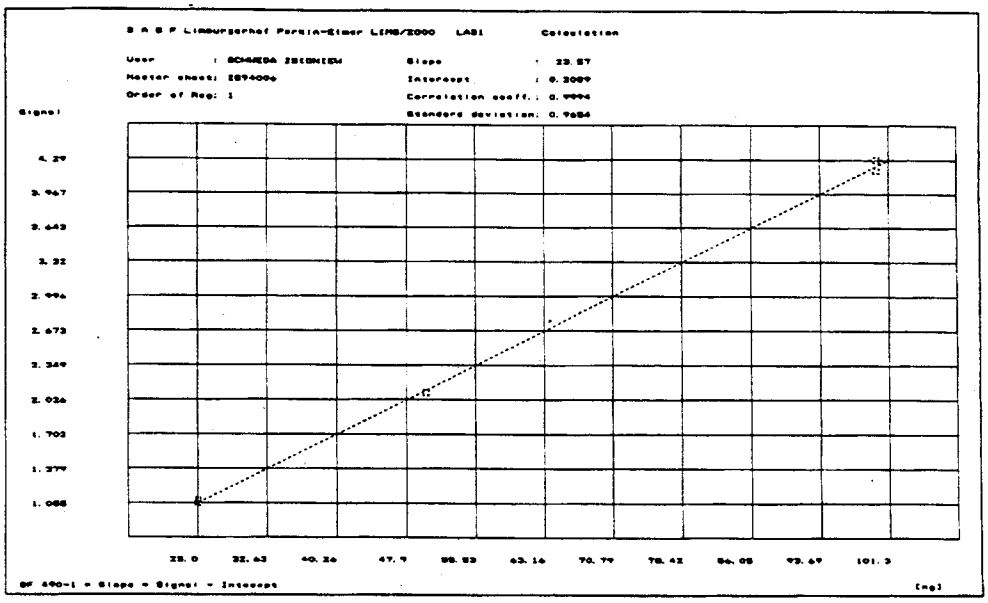


Figure 5: Calibration Curves



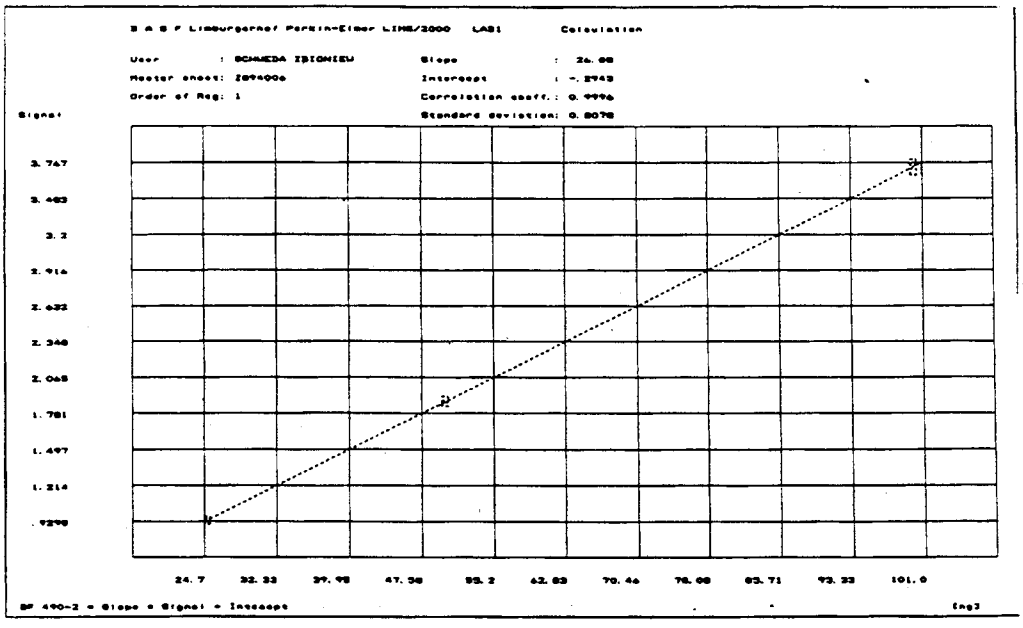


Figure 6: Standard Chromatogram containing 40 ng BF 490-1, BF 490-2 and BF 490-9 each

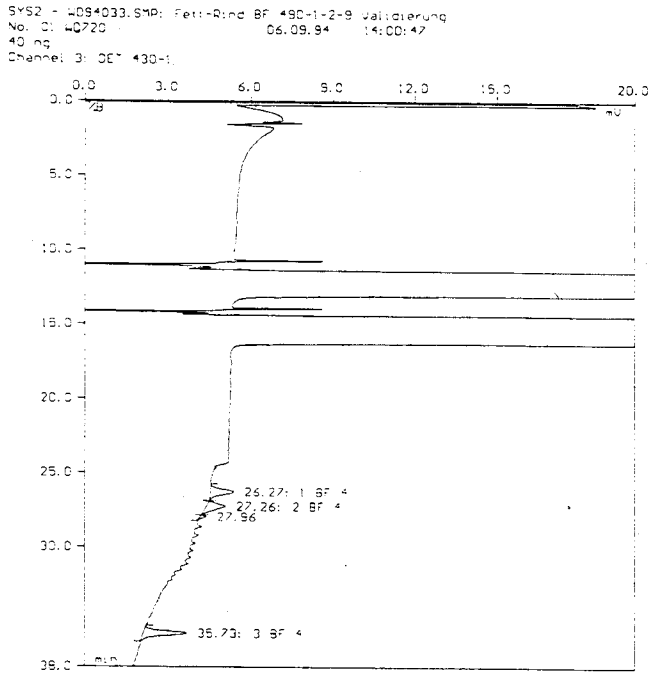


Figure 7: HPLC elution profile of an untreated fat sample

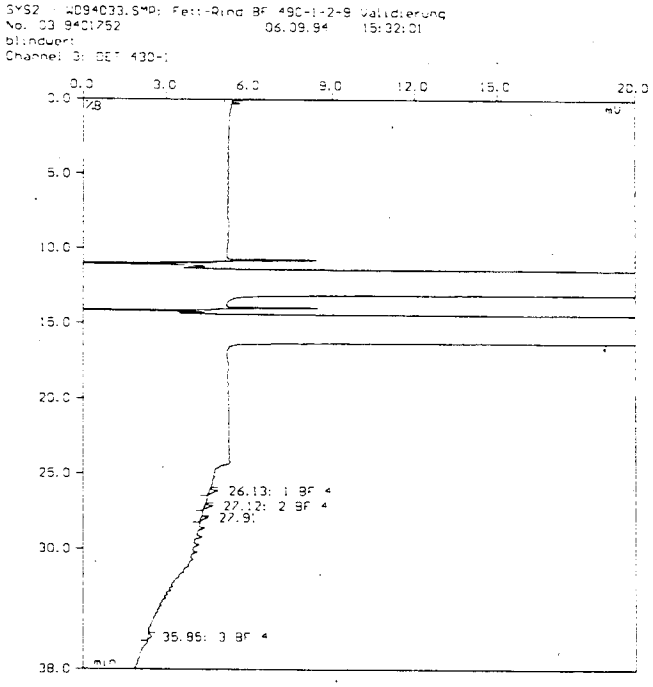


Figure 8: HPLC elution profile of an untreated fat sample fortified with BF 490-1, BF 490-2 and BF 490-9 at a level of 0.010 mg/kg each

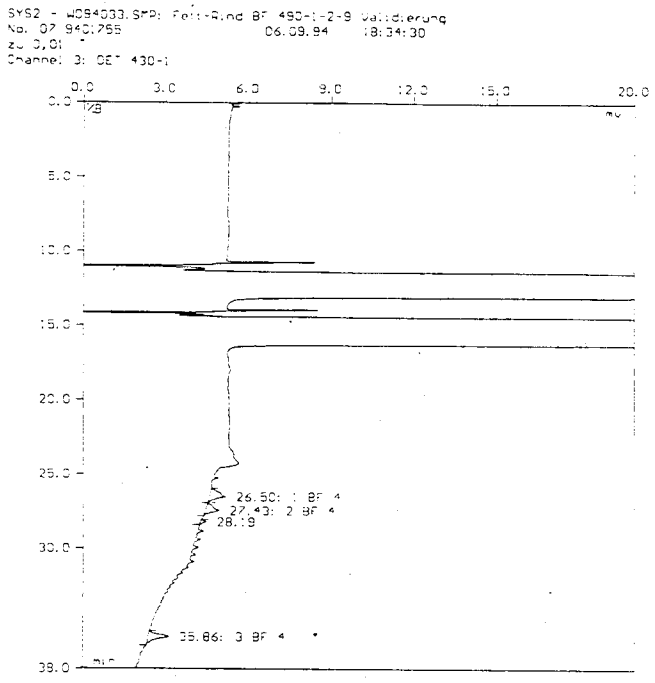


Figure 9: HPLC elution profile of an untreated fat sample fortified with BF 490-1, BF 490-2 and BF 490-9 at a level of 1.00 mg/kg each

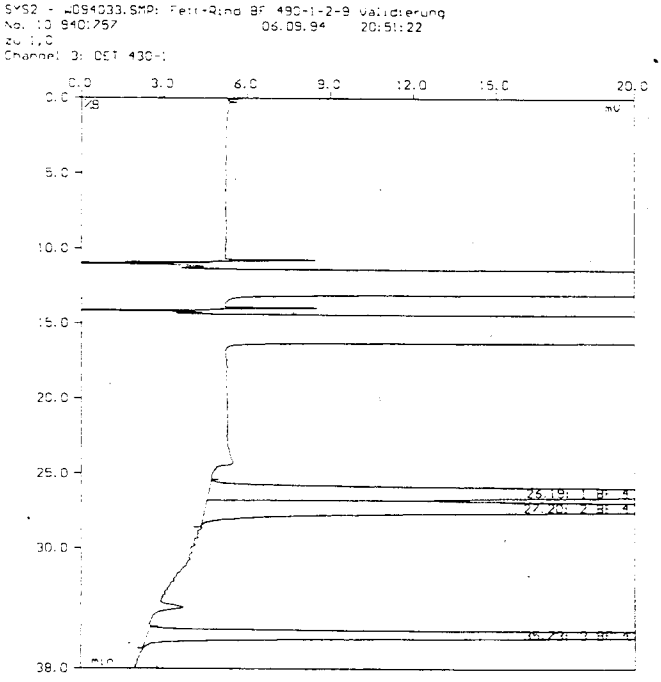
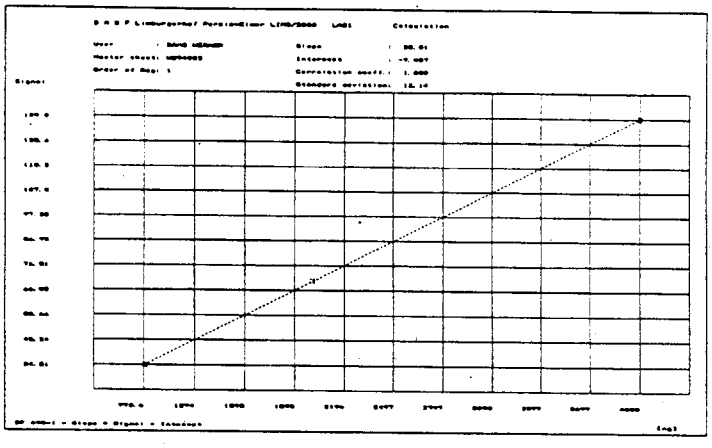
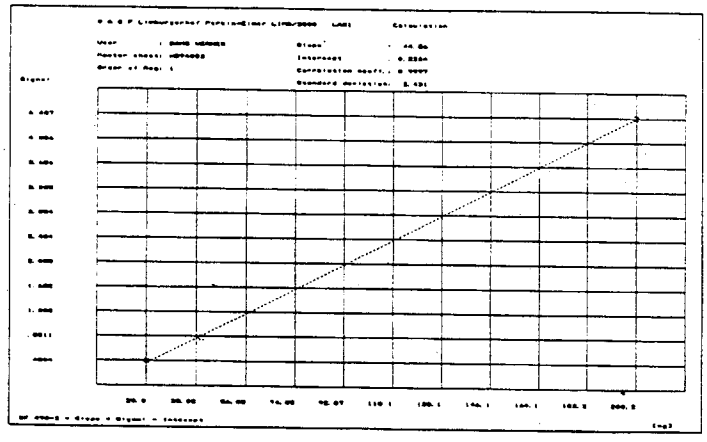
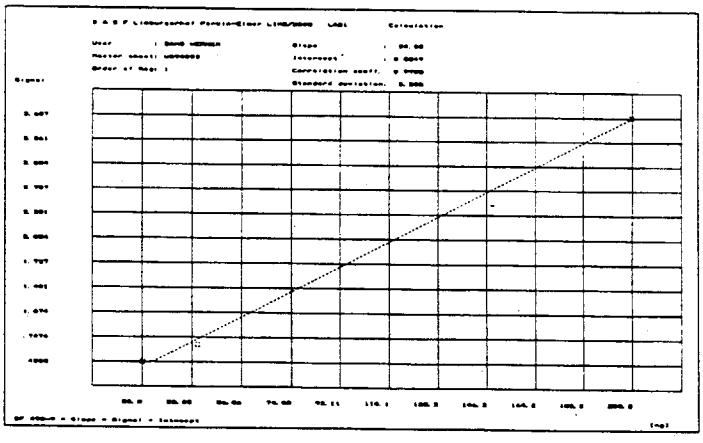


Figure 10: Calibration Curves







EPA ADDENDUM

ACB #B99-73

TMV for Kresoxim-Methyl in Beef Liver

1. ACB recommends that the users **incorporate** the statement on page 33 of 58, titled, 9. Potential Problems, " In case of high content of fat in liver and kidney samples, further extract cleanup steps may be included in the technical procedure.....", and **attach it permanently to item 5.4 page 20 of 58**.

2. Samples are to be suction filtered through a black ribbon filter. It is not known what a black ribbon filter is, so ACB used Whatman's glass microfiber filters # 934-AH. This filter was used in all subsequent filtration steps as well.

3. The method step 5.4 does not specify how long to shake the separatory funnels. ACB shook the funnels for 3 minutes and serious emulsions occurred which required standing for some time in order to breakup the emulsion.

4. The method indicates that samples are to be eluted by gravity from the NH2 SPE column, (step 5.5). The aqueous rinses as well as the elution solvent, did not flow through the column by gravity alone. ACB used slight vacuum in order to elute through these columns.

5. Caution should be taken when evaporating the samples to near dryness as foaming occurs when methanol is evaporated and water remains. This is due to high fat content of certain samples.