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BASF CORPORATION
AGRICULTURAL PRODUCTS GROUP
Agricultural Products Group
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Study Title:

Determination of BAS 490 F and its Metabolites in Pecan

BASF Study Number: 96163

Data Requirement:

Guideline 171-4(c) Residue Chemistry
Residue Analytical Method

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Report Date:

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Performing Laboratory:

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BASF Method Number:

D9611A

This report consists of 77 pages

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PR 86-5 DATA CONFIDENTIALITY CLAIM

No claim of confidentiality 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

Company Agent: Karen R. Blundell

Title: Senior Registration Specialist

Signature: Karen R Blundell Date: June 24, 1997

GOOD LABORATORY PRACTICE STATEMENT

This study meets the requirements for 40 CFR Part 160 Good Laboratory Practices.

Submitter: Karen R. Blundell

Sponsor: [illegible]

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STATEMENT OF THE QUALITY ASSURANCE UNIT

Study Initiation Date: January 06, 1997

The quality assurance unit of the testing facility at the APC has inspected and/or audited the protocol, analyitcal portion including the raw data and the report for this study, and has reported its findings to the study director and to management.

Date of Inspection	Report to study director and to management
01/06/97	01/06/97
01/09/97	01/09/97
01/22/97	01/22/97
05/29/97	05/29/97

Signature of QAU

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Method for Determination of BAS 490 F and its Metabolites in Pecan

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ANALYSES DONE BY: Jesse Thornton and Ira Moss

BASF Method Number D9611A **Report Date:** ***June 24, 1997***

ABSTRACT:

Analytical Method Number D9611 was developed to determine the residues of BAS 490 F and the glycosilated conjugates of BF 490-2 and BF 490-9 in pecan by HPLC. ***Method D9611 was amended to D9611A to make the procedure more clear and to add the optional use of a commercial SPE column. The modifications resulted from an Independent Laboratory Validation of D9611.***. Method development and validation were carried out at BASF Corporation, Research Triangle Park, NC, using representative control pecan.

BAS 490 F , BF 490-B and BF 490-C (glycosilated form of BF 490-2 and BF 490-9, respectively) are extracted by homogenization of the pecan samples in methanol followed by filtration. To this extract an aqueous solution of potassium phosphate (KH_2PO_4) (5 g/L) is added. Aliquots of the extract are reduced to the aqueous phase and after addition of calcium hydroxide ($Ca(OH)_2$) and methanol (MeOH), interfering compounds were precipitated and centrifuged. Supernatant concentration and pH adjustment are performed, prior to the enzymatic hydrolysis in the presence of ascorbic acid. The enzymatic hydrolysis step yields the metabolites BF 490-2 and BF 490-9 in their unbound form. In the following step, BAS 490 F is converted into the metabolite BF 490-1 by hydrolysis with potassium hydroxide (KOH).

The metabolites are separated from matrix interferences in the extract by phase partitioning into dichloromethane (DCM) followed by a solid phase extraction (SPE) amino (NH_2) column clean-up step. This is followed by a second phase partitioning step into DCM. Final detection and quantification is performed by analytical HPLC on a NH_2/C_{18} column combination (column switching). The analytes are detected with a UV-detector at a wave length of 270 nm. The metabolite residues are calculated as BAS 490 F-equivalents.

Recoveries of the analyses averaged 87 ± 11 (n = 11) for BAS 490 F, 90 ± 7 (n = 11) for BF 490-2, 85 ± 6 (n = 11) for BF 490-9, 127 ± 0 (n = 2) for BF 490-B, and 115 ± 1 (n = 2) for BF 490-C. The overall average recovery for all analyses is 91 ± 13 (n = 37).

Study Initiation Date: January 6, 1997
Experimental Start Date: January 9, 1997
Experimental Termination Date: January 23, 1997

1.0 Introduction

BAS 490 F is a new Strobilurin type fungicide used in cereals, apples, citrus, vine, pecan and some vegetables against mildew and rust. Metabolism investigations in apple, grape and wheat show that in all unchanged parent was found. Additionally two metabolites, BF 490-2 and BF 490-9, both present as glycoside-conjugates, were identified to be relevant (References 1-3).

This method was developed at BASF laboratories for the individual determination of BAS 490 F, and the glycosilated conjugates of BF 490-2 and BF 490-9 in pecan.

2.0 Principle of the Method

BAS 490 F, BF 490-B and BF 490-C (glycosilated form of BF 490-2 and BF 490-9, respectively) are extracted by homogenization of the pecan samples in methanol with a polytron for 4 minutes followed by filtration through a Buchner funnel into a 1-L flask.. To this extract an aqueous solution of KH_2PO_4 (5 g/L) is added.

The methanol extract contains the BAS 490 F and the glycosides of BF 490-2 and BF 490-9. A 100 mL aliquot of the methanol extract is reduced to the aqueous phase by rotory evaporation. After addition of $Ca(OH)_2$ and methanol, interfering nonpolar compounds were let to precipitate for 30 minutes. After this period, samples are centrifuged to separate the precipitate. Supernatant concentration and pH adjustment are performed, prior to enzymatic hydrolysis in the presence of ascorbic acid. The surplus of enzyme activity used for this extract facilitates complete and reliable hydrolysis. Ascorbic acid acts both as a preservative and a blocking agent preventing the analytes from adsorption to matrix compounds. The enzymatic hydrolysis step yields the metabolites BF 490-2 and BF 490-9 in their unbound form. In the following step, BAS 490 F is converted into the metabolite BF 490-1 by hydrolysis with KOH.

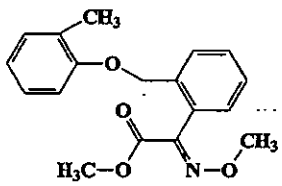
The metabolites are separated from matrix interferences in the extract by phase partitioning into dichloromethane (DCM) followed by a SPE NH_2-column clean-up step. The chromatography step is based on polarity as well as ion-exchange chromatography. It is followed by a second phase partitioning step into DCM to remove ascorbic acid from the extract. Final detection and quantification is performed by analytical HPLC on a NH_2/C_{18}-column-combination (column switching). The analytes are detected with an UV-detector at a wave length of 270 nm.

The metabolite residues are calculated as BAS 490 F-equivalents.

3.0 Structures

BAS 490 F

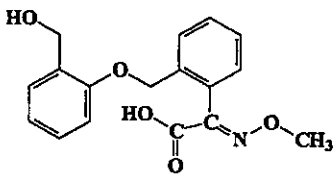
Common name: Kresoxim-methyl (proposed)
Chemical name: Methyl 2-[o-(o-methylphenoxymethyl)phenyl]-2-(methoxyimino) acetate
Structural formula:



Chemical formula: $C_{18}H_{19}NO_4$
Molecular weight: 313.36
Lot No: 39/184-1
Purity: 99.7%

Metabolite BF 490-2

Chemical name: 2-[o-(o-hydroxymethylphenoxymethyl)phenyl]-2-(methoxyimino) acetic acid
Structural formula:

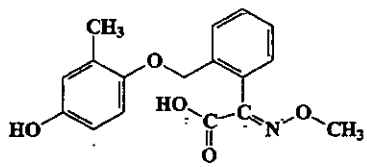


Chemical formula: $C_{17}H_{17}NO_5$
Molecular weight: 315.33
Lot No: 00436-65
Purity: 96.9%

Metabolite BF 490-9

Chemical name: 2-[o-(p-hydroxy-o-methylphenoxymethyl)phenyl]-2-(methoxy-imino) acetic acid

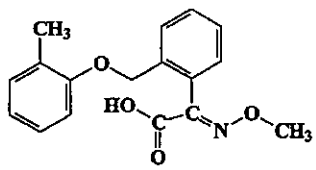
Structural formula:



Chemical formula: $C_{17}H_{17}NO_5$
Molecular weight: 315.33
Lot No: 00436-69
Purity: 99.3%

Metabolite BF 490-1

Chemical name: 2-[o-(o-methylphenoxymethyl)phenyl}-2(methoxyimido) acetic acid
Structural formula:

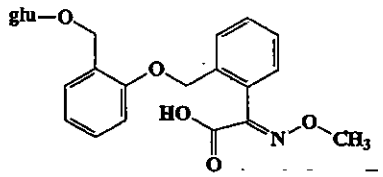


Chemical formula: $C_{17}H_{17}NO_4$
Molecular weight: 299.33
Lot No:___ 00820-251
Purity: 99%

Metabolite BF 490-B (Glucoside of BF 490-2)

Chemical name: 2-[2-(-B-D-glucopyranosyl methylphenoxymethyl) phenyl]-2-methoxyiminoacetic acid

Structural formula:

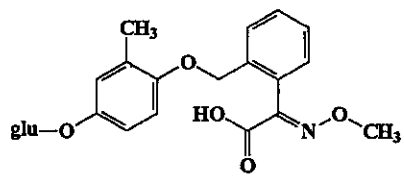


Chemical formula: $C_{23}H_{27}NO_{10}$
Molecular weight: 477.5
Lot No: 1018-89-07
Radio Chemical Purity: 100%
Specific Activity as Parent: 17431 dpm/µg

Metabolite BF 490-C (Glucoside of BF 490-9)

Chemical name: 2-[2-(4-B-D-glucopyranosyl-2-methylphenoxymethyl) phenyl]-2 -methoxyiminoacetic acid

Structural formula:



Chemical formula: $C_{23}H_{27}NO_{10}$
Molecular weight: 477.5
Lot No: 1018-89-18
Radio Chemical Purity: 100%
Specific Activity as Parent: 17431 dpm/µg

4.0 Materials and Methods

4.1 Equipment for Extraction and Sample Clean-up

Glassware and equipment are recommendations only. Equivalent equipment and glassware may be substituted. All glassware ***and glass wool*** should be cleaned with acidic methanol (5% (v/v) of 6M HCl in MeOH) and consequently rinsed with acetone and allowed to dry completely before being used. This procedure is recommended to remove adhering detergents or silica residues from the glassware. Not acid rinsing glassware can have an impact on the recovery of the compounds.

Büchner funnels with adapters	
Filtering Flask	500 mL
Wide neck powder bottle for sample extraction	500 mL
Separatory funnels	125 mL
Flat bottom flasks	500 mL, 250 mL, 125 mL
Graduated Cylinder	100 mL
Erlenmeyer flasks	50 mL
Volumetric flasks	10 mL, 500 mL
Volumetric pipettes	glass, 0.5, 1,2, 4, 5, 10, 100 mL
Plastic, serological, measuring pipettes	10 mL
Disposable, plastic pipettes	3 mL
Homogenizer	Polytron 3035 or Ultra turrax
Glass funnels	5 cm diameter
Cotton wool	Prewashed with DCM,dried at 30°C
Glass wool	***Fisher Scientific***
Test tube, tapered tip	15 mL
PTFE syringe filter	0.45µm
Gastight syringe	25 mL
Whatman #5 Filter paper (Qualitative, 90mm circles)	Cat No. 1005-090
Thick walled glass centrifuge tube with caps	150 mL
pH Meter or pH checker	VWR, Cat. No. 34100-300
Vacuum pump incl. vacuum controller	
Rotary evaporator	
Nitrogen evaporator (N-EVAP)	Labotec, Wiesbaden, FRG
Heated water bath (37°C) with thermo-controller and shaking device	IKA TER 2, Labotec
Ultrasonic water bath	
Automated pipettes, (100 µL and 1000 µL)	Eppendorf or Gilson,
BAKER extraction system complete with taps and vacuum controller	J.T. Baker, Philipsburg, N.Y. Cat. No. 77018-94,
Reservoirs (15 mL) for Baker columns	J.T. Baker, Cat. No. 7119-1
BAKER SPE empty glass column	J.T. Baker, Cat No. 7328-06 ***with 10 micron frit***
BAKER SPE Amino (NH_2) column packing	J.T. Baker, Cat. No. 7028-00
or	
Bakerbond SPE Amino column, 3 mL, 500mg	***J.T. Baker, Cat. No. 7088-03(40 µm, 60A)***
Plastic SPE valve liners	Supelco, Cat. No. 5-7059
Centrifuge (150 mL tube capability)	Beckman or equivalent

Preparation and Preconditioning of BAKER NH_2-columns:

To prepare SPE amino column, fill the empty glass BAKER columns ***(with 10 micron frit at the bottom)*** with 0.5 g each of the NH_2-material mentioned previously. ***Top the packed column with another frit or about one inch height (approximately 150-200 mg) pre-washed glass wool.***
Note: Glass wool should be pre-washed according to Section 4.1 before use.

Pre-packed commercial Bakerbond amino SPE columns can be used instead of manually packing the columns.

Amino columns (hand packed or pre-packed) must be preconditioned. For preconditioning, the column is washed with 2.5 mL of methanol, followed by 2.5 mL of an aqueous solution of KH_2PO_4 (0.1 M, pH 5.0), 2.5 mL of ddH_2O and finally 2.5 mL of methanol. Do not allow the column to go dry at any point while conditioning. Disposable plastic valve liners are to be used on the SPE chamber to limit sample interactions with metal surfaces.

4.2 Reagents

4.2.1 Chemicals

Note:
All chemicals used must be at least of "analytical grade" or must meet equivalent specifications. Suppliers are recommendations only unless otherwise stated.

Methanol	JT Baker Cat No 230-4
Acetone	JT Baker Cat No 010-4
Dichloromethane	JT Baker Cat No 300-4
Acetonitrile	JT Baker Cat No 015-4
Formic acid (conc.)	**[Important! Use only EM Science Cat. No. FX0440-7]**
Ascorbic acid	EM Science Cat No AX1772-1
Calcium Hydroxide, powder	JT Baker, Cat No. 1372-01
KOH 10 mol/L	Make dilution from 45% w solution (molarity 11.7, Aldrich Cat. # 41766-1)
NaCl	VWR Cat No VW6430-5
Na_2SO_4	the pH of its aqueous solution (approx. 5 g in 100 mL ddH_2O) must be 5.5 to 6.5
	JT Baker Cat No 3375-05
KH_2PO_4	JT Baker Cat No 3246-1
β-Glucosidase	Cresent Chemical, Hauppauge, NY, activity 7.1 U/mg, Order No. 22830, Sigma, Cat. No. G-0395, activity 5.7 U/mg
Hesperidinase	St. Louis, MO, Cat. No. H-8137,activity: 7.0 U/g ICN Cat-No. 157339, activity 0.3 U/mg

Water [Milli-Q plus-Grade (>17 MΩ resistivity) or equivalent, in this technical procedure referred to as ddH_2O]

4.2.2 Solutions and Solvent Mixtures

Note: All solvent mixtures and buffers must be prepared by mixing the actual volumes of the individual components.

Solution I "Acidic" water:
880 mg ascorbic acid are made up to 1000 mL with dd H_2O (Must be stored refrigerated. Storage should not exceed one week.)

Solution II Phosphate buffer:
5.0 g of KH_2PO_4 are dissolved in dd H_2O and the volume adjusted to one liter to yield a final concentration of 5 g/L. This will be enough for 40 (10 sample per set) analysis sets. (Stored refrigerated for maximum of one month)

Solution III Ascorbic acid solution:
10 g of ascorbic acid are dissolved in 100 mL ddH_2O to yield a final concentration of 100 g/L.

Note: The ascorbic acid solution (III) must be prepared daily.

Solution IV Saturated NaCl-solution:
NaCl is dissolved in 1000 mL ddH_2O to yield a saturated solution (approximately 350 g).

Solution V Precipitate wash solution:
200 mL methanol is brought to 1000 mL with dd H_2O. (Storage for 2 months)

Solution VI Formic Acid solution (10%):
Add 5 mL concentrated formic acid to 50 mL dd H_2O and mix. Dilute to 100 mL and mix completely. (Storage for 2 month at room temperature)

4.2.3 Enzyme Solution

The following directions are for enzymes with the activities described in 4.2.1.
If enzymes with different activities are used, the weight should be adjusted as needed. Activity of enzymes are defined as unit. One unit will liberate 1.0 µmole of reducing sugar (as glucose) from substrate per minute at a fixed pH and temperature.

Solution VII Hesperidinase and β-Glucosidase stock solution:
200 mg of each dry enzyme is dissolved in 50 mL of water to yield a solution which contains both enzymes in a final concentration of 4 mg/mL of each enzyme.

Note: **50 mL of the enzyme stock solutions are sufficient for the hydrolysis of 20 residue samples. Use an appropriate solution amount for each sample analysis set size based on a 4 mg/mL solution of each enzyme and that 2.5 mL of the enzyme solution is needed for each sample. The enzyme stock solutions must be prepared immediately before use. For short term storage (few hours only) the stock solutions should be kept refrigerated or on ice.**

4.2.4 Solutions for BAKER SPE-columns

Solution VIII NH_2-column elution mixture:
Contains 350 mL ddH_2O (saturated with NaCl, solution IV) + 12 mL formic acid (conc.) + 10.5 g ascorbic acid + 50 mL methanol + 100 mL acetonitrile.

After preparation, this solution must be kept for a minimum of 24 hour at approximately +4°C prior to use to allow surplus NaCl to precipitate. After precipitation, the supernatant must be filtered before it can be used in the method. The solution must be stored in a tinted glass bottle, refrigerated for maximum 2 months.

4.2.5 Stock and Spiking Solutions

Stock solutions with analyte concentrations of 1 mg/mL each are prepared for BAS 490 F in acetone and for BF 490-2, BF 490-9 and BF 490-1 in acetonitrile.

BF 490-2 and BF 490-9 are representative of their glycoside conjugates for fortification purposes. From the stock solutions the spiking solution for BAS 490 F, BF 490-2 and BF 490-9 are prepared with final analyte concentrations of 1.25, 6.25 and 25 mg/mL by dilution with appropriate volumes of acetonitrile. These concentrations are recommended and may be modified as appropriate.

All stock and spiking solutions must be stored at approximately 4°C in the dark. Eventhough, stock solutions have a longer shelf life, it is recommended that stock solutions were made fresh every 30 days (references 4-6).

4.2.6 Standard Solutions for Calibration

The stock solutions specified in 4.2.5 may be used for the preparation of calibration standards.

Standards of BF 490-2, BF 490-9 and BF 490-1 for column testing and calibration of the analytical HPLC: From the 1 mg/mL stock solutions in acetonitrile, working standards (25 µg/mL) were made. From working standards, calibration standards are prepared in a mixture of acetonitrile + "acidic" water (Solution I) (10 + 90) with final analyte concentrations in the range of 0.025 to 1.25 µg/mL (or higher if required).

All solutions used for calibration must be stored at approximately +4°C in the dark for no longer than 24 hours prior to use.

4.2.7 **Buffers for HPLC Separation**

For analytical HPLC:

Solution IX Pre-column elution buffer:
Contains ddH_2O + acetonitrile + formic acid (conc.) 90 + 10 + 1.0* (v/v/v)

***Note: The volume of formic acid may need to be adjusted to the performance of the NH_2-precolumn. The performance must be evaluated by test injections prior to analysis. All three compounds should be eluted from amino column within 4-15 minutes after injection.**

Solution X Analytical HPLC elution buffer A:
Contains ddH_2O + acetonitrile + formic acid (conc.) 90 + 10 + 0.5 (v/v/v)

Solution XI Analytical HPLC elution buffer B:
Contains ddH_2O + acetonitrile + formic acid (conc.) 10 + 90 + 0.5 (v/v/v)

Note: All elution buffers specified in 4.2.7 must be degassed or saturated with helium for approximately 10 minutes, or by equivalent treatments prior to use.

4.3 **Instrumentation and Chromatography Conditions**

4.3.1 **Analytical HPLC with Column Switching**

3 HPLC - pumps	
HPLC-Detector	UV/visible, fixed or variable wavelength
Autosampler	With 200 µL loop
Switching valves	Rheodyne

Precolumn specification:
NH_2- Nucleosil (5µ, 100 Å),100 x 4.0 mm (internal diameter), Phenomenex, USA
Catalog No. 00D-0327-D0

Separation column specification:
C_{18}-Nucleosil (5µ, 100 Å) 250 cm x 3.2 mm (internal diameter), Phenomenex, USA
Catalog No. 00G-0323-R0

Note: The equipment listed in 4.1 - 4.3 may be substituted by chemicals and instruments with similar specifications. HPLC columns with equivalent stationary phases and similar specifications may be available from other sources. If the use of materials with specifications other than those stated is intended, applicability of the new equipment for this method must be confirmed.

Note: The following procedures should be followed for cleaning and backflushing the NH_2 pre-column and the C_{18} analytical column.

NH_2 pre-column

This procedure should be performed after 20 sample injections or when the column-switching windows are not consistent during an analysis set.

1. Reverse the direction of the NH_2 pre-column on the HPLC and make sure that the exit line will flow into waste and not into the C_{18} column.
2. Replace Solution IX (Pre-column elution buffer) with a solution of ACN:dd$H_2$0:Formic Acid (90:10:1.5)(v/v/v).
3. Pump this solution through the NH_2 pre-column at 1.0 mL/min for 30-60 minutes.
4. Stop the column flow, reverse the column to its original position and equilibrate the column with Solution IX.
5. Refer to Section 5.3.7 to check the column-switching windows.

C_{18} analytical column

This procedure should be performed after 50 sample injections or when ever peak shape becomes compromised.

1. Reversed the direction of the C_{18} analytical column on the HPLC.
2. Replace Solution XI (Analytical HPLC elution buffer B) with a solution of ACN:dd$H_2$0:Formic Acid (90:10:1.5)(v/v/v).
3. Pump this solution through the C_{18} analytical column at 1.0 mL/min for 30-60 minutes.
4. Stop the column flow, reverse the column to its original position and replace wash solution with solution B (solution XI). Equilibrate the column with Solution X.

5.0 Analytical Procedure

5.1 Fortification of Samples with BAS 490 F and its Metabolites for Recovery Experiments

25 g of untreated sample material is weighed into a wide neck glass jar (500 mL). Fortification solutions with BAS 490 F, BF 490-2 and BF 490-9 at appropriate concentrations are added to the samples. The correlation between the concentration of the spiking solution and the corresponding final analyte concentration in the sample is shown below. Other fortification volumes and standard concentrations may be used as needed.

Sample Weight	Concentration of spiking solution	Volume of spiking solution	Level of Fortification
25 g	1.25 mg/mL	1.0 mL	0.05 mg/kg (*)
25 g	6.25 mg/mL	1.0 mL	0.25 mg/kg
25 g	25.0 mg/mL	1.0 mL	1.00 mg/kg

(*) Proposed limit of quantitation of the method.

5.2 Extraction of the Sample Material

25 g sample material is weighed into a wide neck glass jar (500 mL) and 1.0 mL of ascorbic acid solution (III) is added. The sample is homogenized in 250 mL of methanol for 4 minutes using an Ultraturrax or a Polytron at a speed sufficient to homogenized the sample. The homogenate is filtered under vaccum through a Whatman #5 filter paper and a Büchner funnel (9 cm diameter) into a suction bottle (1000 mL). The residual solids retained in the filter are washed with small methanol rinses. (Make sure that the total volume of MeOH does not exceed 350 mL). 100 mL of an aqueous solution of KH_2PO_4 (5 g/L) are added into a 500 mL volumetric flask. The combined methanol filtrates are quantitatively transferred to this flask with methanol rinses. The contents of the flask are thoroughly mixed. The total volume is made up to 500 mL with methanol and the contents are mixed once more.

5.3 Clean-up and Hydrolysis

5.3.1 Sample Preparation prior to Enzymatic Hydrolysis

A 100 mL aliquot of the aqueous methanol extract (20% aliquot) is transferred into a preweighed 500 mL flat bottom flask (the large flask is needed to reduce the risk of bumping during evaporation). The extract is reduced to a volume of approximately 20 mL by rotary evaporation (under vacuum, 45±5°C) and adjusted to a total mass of 25 g with ddH_2O. 2.5 g calicum hydroxide followed by 12.5 mL methanol is added to the flask, (rinse any $Ca(OH)_2$ powder from neck of the flask with this methanol). The flask is capped and then shaken vigorously for 30 seconds. Allow the flask to sit for 30 minutes with occational mixing (every 10 minutes).

Quantitatively transfer sample to 150 mL centrifuge tube with about 3 rinses of 2-3 mL Solution V (20% MeOH). The original sample flask should be clear of any $Ca(OH)_2$ precipitate at this stage. Cap each sample and place tubes in centrifuge being sure to check the counter-weight of each pair of tubes. Spin tubes at 4000 rpm for 10 minutes. Carefully remove tubes from centrifuge and decant sample back into original 500 mL flat-bottom flask. Make sure that no precipitate is poured back into sample flask. Add 20 mL Solution V to each sample, cap, vortex and sonicate, about 10 seconds so that sample is mixed thoroughly. Centrifuge at 4000 rpm for 10 minutes. Decant supernatant into original sample flask. Add another 20 mL of the solution V to each sample and repeat the same procedure.

Add 0.5 mL 10% Formic acid (Solution VI) to dissolve any precipitate and to reduce the chance for sample bumping during rotary evaporation. Reduce the sample volume to approximately 10 mL by rotary evaporation (under vacuum, 45±5°C) and adjust the total mass to 20.0 g with ddH_2O (extract must be absolutely methanol free).

NOTE: **20.0 gram is an aliquot weight, therefore care should be taken to ensure accuracy.**

NOTE: **The sample at this point is very suseptible to bumping. Start the evaporation at or above 250 torr and bring the vacuum down by 35-50 torr every 5 minutes being very careful to watch each flask. Eventually bring the vacuum down to < 50 torr to evaporate off the water.**

If required, the flask should be sonicated with an ultrasonic bath for 10-15 seconds after adjusting the mass, to dissolve all residues on the flask.

5.3.2 Enzymatic Hydrolysis of Analyte Glycosides

A 10.0 mL aliquot of the final aqueous phase from 5.3.1 (20.0 g) is transferred into a 50 mL Erlenmeyer flask or equivalent using a 10 mL plastic pipette. The pH of the solution is adjusted to 3.75±0.25 with approximately 0.5 mL solution VI (10% formic acid). Check the pH with pH checker and adjust, if required. To this flask, 0.5 mL of the ascorbic acid solution (III) and 2.5 mL of the Glucosidase and Hesperidinase stock solution (VII) are added. Each measuring flask is closed with a glass stopper and subsequently incubated at 37°C in a waterbath equipped with a magnetic stirring device or a shaking water bath. Incubation proceeds over night (or approximately 14 hours) with slow but constant agitation.

5.3.3 Alkaline Hydrolysis to Convert BAS 490 F into BF 490-1

Upon completion of the enzymatic hydrolysis the reaction mixture of 5.3.2 is adjusted to approximately pH 13 by adding 250 µl of 10 mol/L KOH. This hydrolysis of BAS 490 F proceeds in 1 hour at room temperature.

5.3.4 Phase Partitioning with Dichloromethane

Upon completion of hydrolysis, the reaction mixture of 5.3.3 is adjusted to pH 2 to 2.2 with concentrated formic acid (approximately 2 mL)

Note: Final pH must be checked with a calibrated pH meter or pH checker calibrated to ensure the accuracy of the pH. Be sure to rinse the pH meter with 1-2 mL of water into the sample that is being checked before proceeding to the next sample.

The acidified phase is quantitatively transferred into a 125 mL separatory funnel with 3-5 mL DCM rinses. The flask is then rinsed twice with 10 mL of DCM each and the DCM added to the separatory funnel. Shake the funnel for about 1 min. A glass funnel containing a plug of DCM prewashed cotton wool is overlayed with approximately 10 g Na_2SO_4, which is also prewashed with DCM, and placed over a 125 mL (or equivalent) flat bottom flask. The bottom DCM phase from the separatory funnel is passed through the Na_2SO_4 and collected into the flask. After the first extraction, the pH must be checked and, if necessary, readjusted to 2-2.2 with formic acid.

Repeat the extraction twice more with the addition of 20 mL of DCM. Add each 20 mL DCM aliquot first to the original sample flask and then to the 125 mL separatory funnel. This will ensure complete transfer of the sample to the separatory funnel. Some times emulsion can be formed during DCM partitioning. In this case first allow separatory funnel to sit for 5 minutes, then gently swirl the funnel while holding upright to break emulsion. This may have to be repeated after all each addition of DCM. If the emulsion still exists, centrifuge the sample for 5 minutes at 2000 rpm to break the emulsion. Each DCM layer will be passed through the Na_2SO_4 layer and collected in the same flask. After the third extraction, rinse the Na_2SO_4 layer with 5-10 mL DCM into the sample collection flask. The water-free DCM filtrates are subsequently reduced to dryness by rotary evaporation (vacuum approximately 600 mbar, 45±5°C) DO NOT OVERDRY UNDER

VACUUM. If any DCM residue is left in the flask dry it with a low stream of nitrogen for a few seconds.

5.3.5 SPE NH_2-column Clean-up

A reservoir is connected to a BAKER SPE NH_2-column preconditioned as outlined in 4.1. The dry residue from 5.3.4 is redissolved in 5.0 mL methanol. In some matrices sonication for a few seconds may facilitate this process. Exactly 4.0 mL of this solution are pipetted into the reservoir and percolated through the column.
NOTE: Sample must come out of column drop by drop. No vacuum is required for hand packed SPE columns with glass wool on the top, but a very slight vacuum may be required to start the flow in pre-packed SPE columns.
The column is washed with 2.5 mL methanol after which the column is suction dried for about 10 to 20 seconds. Both the pre-eluate and the column wash solution are discarded. A 10 mL flask or equivalent is placed into the vacuum box for collection of the sample. 1 mL ddH_2O is placed onto the column, the dried column is infiltrated with the water and then collected in the sample flask. Be careful not to allow the column to run dry at this point. 10 mL of the NH_2-column elution mixture (VIII) is used to elute the sample into the collection flask. The total volume of the sample after this step will be 11 mL. All of these steps are done under vacuum with a flow of 0.5 to 1.0 mL/min.

5.3.6 Phase Partitioning with Dichloromethane

With two rinses of 5 mL ddH_2O each, the eluate of 5.3.5 is transferred into a 125 mL separatory funnel. The extract is acidified to a pH of 2 to 2.2 with 0.5 mL of concentrated formic acid. The final pH must be checked with a calibrated pH meter or calibrated pH checker to ensure the accuracy of the pH. Be sure to rinse the pH meter with 1-2 mL of water into the sample that is being checked before proceeding to the next sample.

The sample is extracted three times with 20 mL each of dichloromethane. Add each 20 mL DCM aliquot first to the original sample flask and then to the 125 mL separatory funnel. This will ensure complete transfer of the sample to the separatory funnel. If emulsion forms during DCM partitioning, allow separatory funnel to sit for 5 minutes, then gently swirl the funnel while holding upright to break emulsion. If the emulsion still exists, centrifuge the sample for about 5 minutes at 2000 rpm to break the emulsion. A glass funnel containing a plug of DCM prewashed cotton wool is overlayed with approximately 10 g Na_2SO_4, which is also prewashed with DCM, and placed over a 125 mL (or equivalent) flat bottom flask. The bottom DCM phase after each extraction is passed through the Na_2SO_4 and collected into the same flask and subsequently reduced to approximately 1 mL by rotary evaporation (vacuum approximately 600 mbar, 45±5°C). After the first extraction, the pH must be checked and, if necessary, readjusted to 2-2.2 with formic acid

The 1 mL of DCM is transferred into a 10 mL tapered test tube using plastic transfer pipettes with 3 additional 1 mL rinses of DCM. The DCM is evaporated using a nitrogen evaporator connected to a water bath at 45±5°C. Before the sample reaches complete dryness, rinse the sides of the test tube with approximately 1 mL DCM to ensure that no sample remains on the sides of the test tube. Proceed with the evaporation and remove the sample as soon as it reaches dryness.

5.4 Quantification of BF 490-2, BF 490-9 and BF 490-1 by analytical HPLC

The dried sample is redissolved by adding 100 µL acetonitrile, sonicating the sample for a few seconds using an Ultrasonic bath and then mixing on a vortex. 900 µL acidic ddH_2O (pH 3.4, see 4.2.2) is then added and mixed by using a vortex.

Centerfuge or filter the sample through a 0.45 µm PTFE filter prior to injection to improve chromatography and stabilize the retention time of the analytes.

The final volume can be changed to 2.0 mL (200 µL acetonitrile and 1800 µL ddH_2O) depending on the sensitivity of the UV detector. For analysis, 200 µL of this solution is injected into a HPLC with the aid of an autosampler.

Separation of the metabolites from matrix interferences on the NH_2-precolumn is performed by isocratic elution with 10% acetonitrile in ddH_2O and 1.0% formic acid (*Pre-column elution buffer (IX)* at a pH of approximately 2.2 and at a flow rate of 0.7 mL/minute. BF 490-9 is collected onto the analytical C_{18}-column in a fraction typically collected from approximately 6.9 to 8.15 minutes. This window can be varied from 5 to 9 minutes, depending on the condition of the amino column. The time it takes to transfer BF 490-9 from the amino column to C_{18} column is usually about 1.2 minutes. BF 490-2 and BF 490 1 are collected onto the analytical C_{18}-column in a fraction from approximately 9.1 to 11.3 minutes after injection. This window can also be changed, depending on the condition of the amino column and the pH of the mobil phase. BF 490-2 and BF 490-1 may be eluted together as one broad peak, or as two separated peaks. Separate injection of these two metabolites can be use to confirm their co-elution. When they elute together, the area is usually twice the area for BF 490-9 (with the same concentration) or twice the area for BF 490-2 or BF 490-9, when they injected separately. Typical chromatograms from the amino column profile are shown in the next two pages.

The fractions containing the metabolites are loaded onto the analytical C_{18}-column using a stop flow technique. The exact switching windows must be determined for each HPLC system prior to injection of samples. This should be performed by replacing the C_{18} column with an empty capillary tube (0.12-0.17mm id) and injecting a 100 or 250 ng standard onto the NH_2 column and eluting the analytes with an isocratic elution of 0.7 mL/min *Pre-column elution buffer (IX)* directly into the detector. This system should be set-up to check the column-switching windows after every new batch of amino column mobile phase. The windows can be modified as needed according to the retention time of the 3 analytes on the NH_2 column injection.

Analysis of the metabolites on the C_{18}-analytical column is performed with a gradient of acetonitrile from 10% [*Analytical HPLC elution buffer A (X)*] to 90 % acetonitrile *[Analytical HPLC elution buffer B (XI)]* at a flow rate of 0.7 mL/minute. The metabolites are quantified by UV-detection at a wavelength of 270 nm.

A typical control program for the analytical HPLC separation with NH_2/C_{18}-column switching and stop flow technique as well as column switching valve configuration are shown on the next few pages.

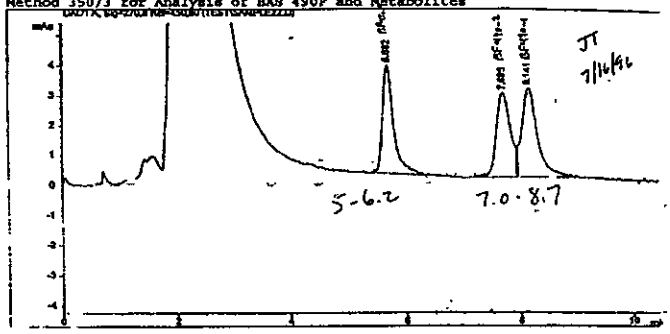
Typical amino column profile for 100 ng injection of BF 490-9, BF 490-2 and BF 490-1. BF 490-1 and BF 490-2 were partially separated.

Data File C:\HPCHEM\1\DATA\TEST\SAMPLE22.D Sample Name: test

100 ng test

Injection Date : 7/16/96 2:48:42 PM
Sample Name : test Vial : 100
Acq. Operator : jthornton Inj Volume : 200 µl

Method : C:\HPCHEM\1\METHODS\490AN.M
Last changed : 7/3/96 1:29:52 PM by jthornton
Method 350/3 for Analysis of BAS 490F and Metabolites



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Sample Amount : 1.00000 [ng/ul] (not used in calc.)

Signal 1: DAD1 A, Sig=270,8 Ref=450,80
Results obtained with enhanced integrator!

Peak #	RetTime [min]	Type	Width [min]	Area [mAu*s]	Height [mAu]	Area %	
1	5.682	BV	0.1981	48.67891	3.62839	31.0732	BF 490-9
2	7.695	BV	0.2320	48.82485	2.84806	31.1664	BF 490-2
3	8.141	VB	0.2165	59.15500	2.99995	37.7604	BF 490-1
Totals :				156.65876	9.47640		

*** End of Report ***

Instrument 1 7/16/96 2:59:24 PM jthornton Page 1 of 1

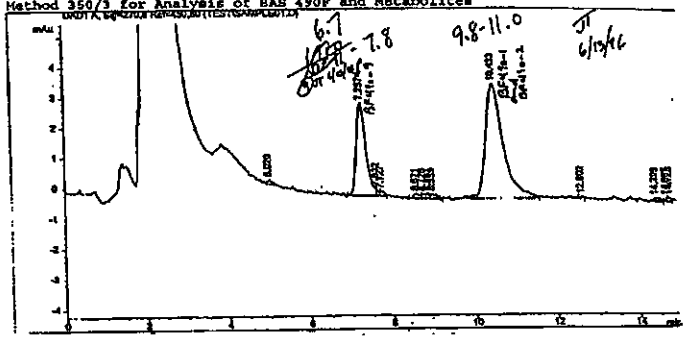
Typical amino column profile for 100 ng injection of BF 490-9, BF 490-2 and BF 490-1. BF 490-1 and BF 490-2 were co-eluted.

Data File C:\HPCHEM\1\DATA\TEST\SAMPLE01.D Sample Name: test

pre-column test injection

Injection Date : 6/13/96 10:33:52 AM
Sample Name : test Vial : 100
Acq. Operator : jthornton Inj Volume : 200 µl

Method : C:\HPCHEM\1\METHODS\490AM.M
Last changed : 6/12/96 3:22:24 PM by jthornton
Method 350/3 for Analysis of BAS 490F and Metabolites



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Sample Amount : 1.00000 (ng/ul) (not used in calc.)

Signal 1: DAD1 A, Sig=270,8 Ref=450,80
Results obtained with enhanced integrator!

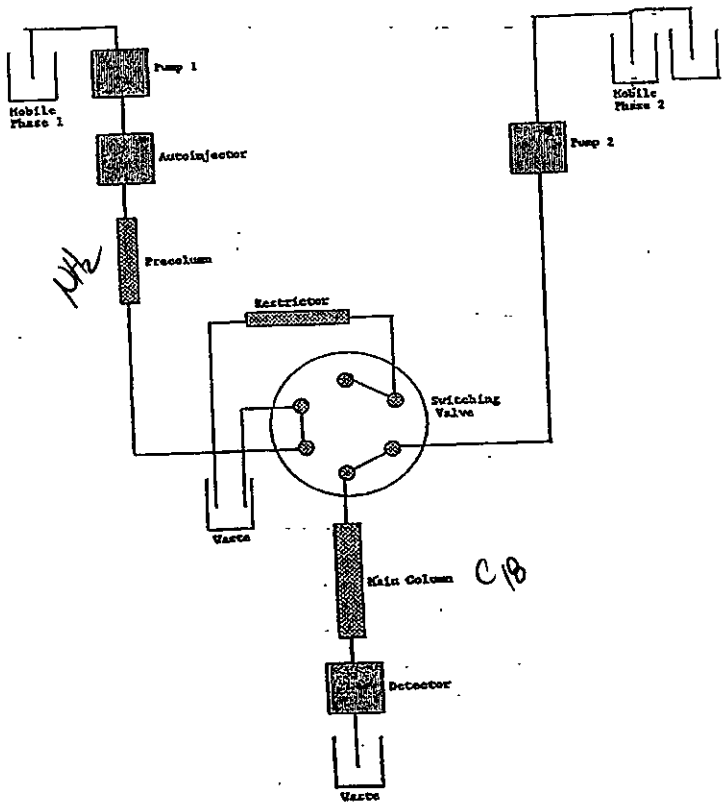
Peak #	RetTime [min]	Type	Width [min]	Area [mAu*s]	Height [mAu]	Area %	
1	5.020	PB	0.0769	9.26227e-1	1.68570e-1	0.5165	
2	7.257	PV	0.2190	49.24270	3.07213	28.5238	BF490-9
3	7.622	VB	0.0626	9.32713e-1	2.48208e-1	0.5403	
4	7.727	BP	0.0820	1.04071	1.60590e-1	0.6028	
5	8.571	PV	0.1272	1.48572	1.60434e-1	0.8606	
6	8.726	VV	0.0607	7.25124e-1	1.58769e-1	0.4200	
7	8.861	VV	0.0763	8.41148e-1	1.75465e-1	0.4872	
8	8.969	VP	0.1057	1.33170	1.67327e-1	0.7714	
9	10.433	VV	0.3640	113.38699	3.80094	65.6804	BF490-2 & BF490-1

Typical column-switching and gradient profile program for analytical HPLC

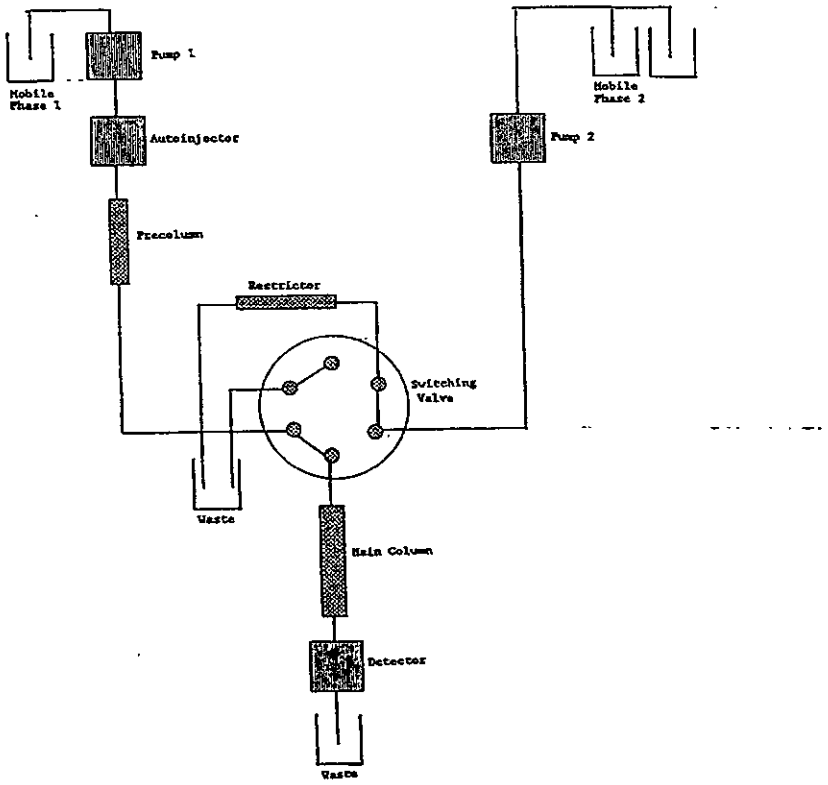
Under these conditions metabolites BF 490-2, BF 490-9 and BF 490-1 will elute at approximately 27.2, 28.1 and 32.6 minutes respectively.

Time (min)	Pre-Column Isocratic Pump		Analytical Gradient Pump		
	Flow (mL/min)	Collection Window	Flow (mL/min)	Buffer A (%)	Buffer B (%)
0.0	0.7		0.7	100	0
6.9		On			
7.2			Stop		
8.1		Off			
9.1		On			
9.9			0.7	100	0
10.3		Off			
10.5				100	0
34.0				30	70
35.0				0	100
37.0				0	100
37.25				100	0
50.0				100	0

Column switching valve configuration. Configuration for elution of BAS 490 F metabolites from amino column (precolumn) or C_{18} column (main column).



Column switching valve configuration. Configuration for transfer of BAS 490 F metabolites from amino column (precolumn) to C_{18} column (main column).

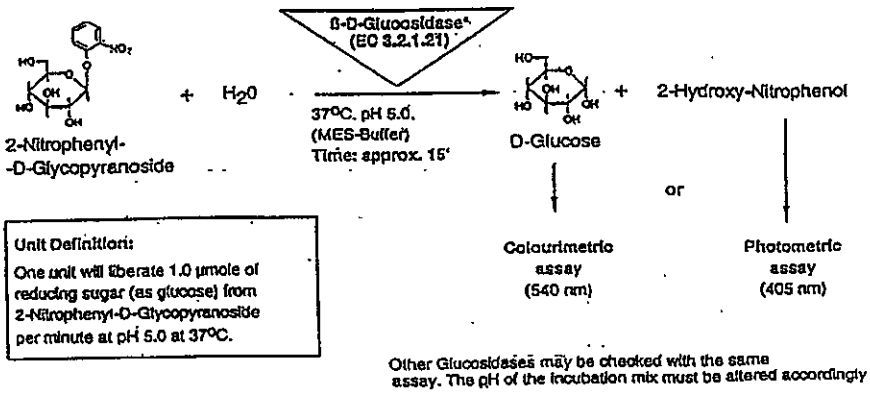


5.5 Test of Enzyme Activity

Enzymatic hydrolysis of the glycosides of BF 490-2 and BF 490-9 with β-Glucosidase and Hesperidinase as outlined in this method was adopted from the metabolism studies for BAS 490 F. Surplus of enzymes in the incubation mix as well as the prepurification of the extracts allow the complete and reliable hydrolysis of the conjugates. Both enzymes are widely available from various suppliers. The enzymatic activity in each batch is specified and guaranteed by the suppliers (for example 4.2.1) if the required storage conditions are maintained. However, it may be useful to check the activity of both enzymes in the analytical laboratory itself.

Fast and reliable biochemical assays are available to test the activity of both enzymes. The biochemical reactions are shown in the following figures. Methods for the quantitative assays for hesperidnase and β-Glucosidase are shown in attachments 1 and 3, respectively. Shorter qualitative TLC assays are shown for both enzymes in attachments 2 and 4 respectively.

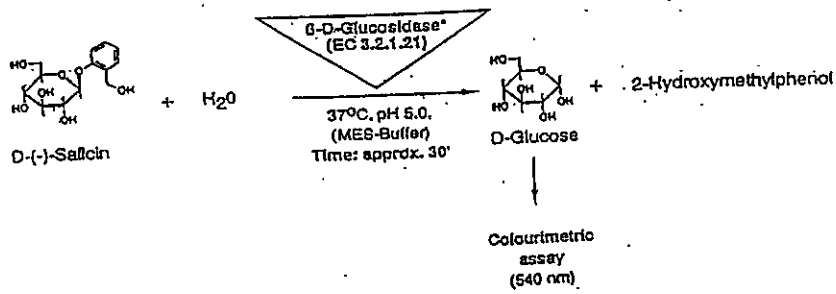
Biochemical Assay for β-Glucosidase with 2-Nitrophenyl-D-Glycopyranoside



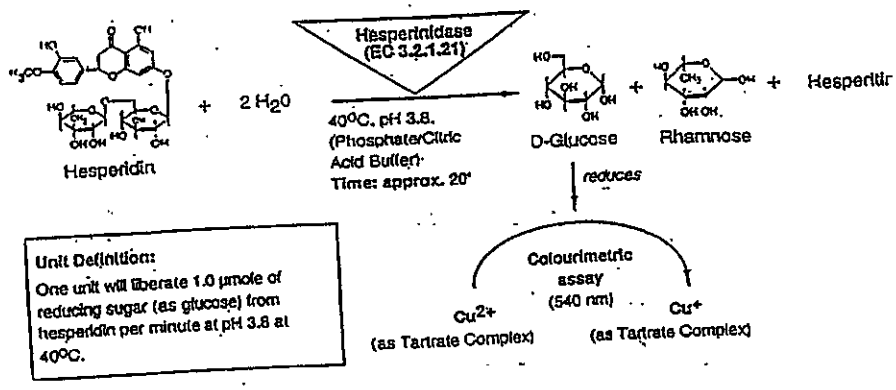
Unit Definition:
One unit will liberate 1.0 µmole of reducing sugar (as glucose) from 2-Nitrophenyl-D-Glycopyranoside per minute at pH 5.0 at 37°C.

Other Glucosidases may be checked with the same assay. The pH of the incubation mix must be altered accordingly

Biochemical Assay for β-Glucosidase with D-(-)-Salicin



Biochemical assay for Hesperidinase with Hesperidin



The protocol and chemicals required for the biochemical assay and the description for a fast TLC method for both enzymes is described in detail in Attachments 1-4.

6.0 Methods of Calculation

6.1 Calibration Procedures

Calculation of results is based on peak height measurements using a calibration curve. To obtain a standard curve, 200 µL of three to four different standard concentrations, for example 50, 125, 500 and 1250 ng/mL of a BF 490-1, BF 490-2 and BF 490-9 are injected. These correspond to 12, 25, 100 and 250 ng of each analyte, respectively. The peak height for each analyte is plotted versus the amount of injected standard (ng).

6.2 Analyte in Sample

6.2.1 Principle

Calculation of results is based on peak height measurements. The amount of BF 490-1, BF 490-2 and BF 490-9 in injected samples is determined from the calibration curve and the equation described in 6.2.2 which is utilized for the determination of the residue (R). Calculation can also be made by a suitable computer program.

6.2.2 Calculation of Residues

The concentrations of BAS 490 F, BF 490-2 and BF 490-9 in the sample material are calculated as shown in equation

1.a $$TR_{BAS\ 490\ F} = F \times \frac{V_{end} \times C_{BF\ 490\text{-}1}}{V_i \times S_m} \times \frac{100\%}{A}$$

1.b $$TR_{BF\ 490\text{-}2} = \frac{V_{end} \times C_{BF\ 490\text{-}2}}{V_i \times S_m} \times \frac{100\%}{A}$$

1.c $$TR_{BF\ 490\text{-}9} = \frac{V_{end} \times C_{BF\ 490\text{-}9}}{V_i \times S_m} \times \frac{100\%}{A}$$

TR	=	Total residues in the sample [mg/1000 g sample material]
Vend	=	End volume of the extract after all dilution steps [mL]
$C_{BF\ 490}$	=	Amount of analyte in the injection volume as read from the calibration curve [ng]
Vi	=	Extract volume injected into the HPLC (aliquot of V_{end}) [µL]
Sm	=	Weight of the plant sample [g]
A	=	Aliquot [%] of the total extract which is used in V_{end} = 8 %
F	=	Molecular weight correction factor to convert BF 490-1 to BAS 490 F = 1.047

For routine analyses residue, results should not be corrected for procedural recoveries. Results of fortification experiments should be listed individually.

6.2.3 Metabolite results expressed as BAS 490 F equivalents

The metabolite residue concentrations as calculated in the equations 1.b and 1.c represent the absolute mass of the analyte in a sample. If these residues are to be expressed as equivalents of BAS 490 F the molar mass ratios of BAS 490 F to either BF 490-2 and BF 490-9 must be considered. This ratio of 1 to 1.006 is identical for both metabolites.
The metabolite residue concentrations expressed as parent equivalents are calculated as shown in equations 2.a and 2.b.

2.a $$TR_{PE} = \frac{TR_{BF\ 490\text{-}2}}{1.006}$$

2.b $$TR_{PE} = \frac{TR_{BF\ 490\text{-}9}}{1.006}$$

TR_{PE} = Residues expressed as BAS 490 F equivalents

6.3 Calculation of Recovery

Correct fortification results for residues found in the control sample as follows:

TR_{PE} (corrected) = TR_{PE} (in fortified control) - TR_{PE} (in control)

Determine percent recovery of analyte from the fortification samples as follows:

$$\% \text{ Recovery} = \frac{TR_{PE} \text{ (corrected)} \times 100}{TR_{PE} \text{ (analyte added)}}$$

7.0 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 approximately 2 working days (16 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. After the first day of analysis the samples should be undergoing enzyme hydrolysis. This will proceed overnight (approx. 14 hours). The second day, the samples should be processed to completion.

A time management protocol which proved to be optimal for method D9611 is shown below:

Sample Material	
Spiking	
Extraction	**Day 1**
Extract clean-up: BAS 490 F, BF 490-2, BF 490-9	
Enzymatic Hydrolysis of BF 490-2 and BF 490-9 Conjugates	
Alkaline Hydrolysis of BAS 490 F	
Extract clean-up steps	**Day 2**
Test analytical HPLC - Pre-column Testing -	
Analytical HPLC NH_2/C_{18}-Columns	
Calculation of data	**Day 3**

8.0 Results and Discussion

8.1 Accuracy and Precision of Validation Results

Subsamples of control pecan nutmeat were fortified with 0.05, 0.025 and 1.0 ppm levels of BAS 490 F, BF 490-2 and BF 490-9. BF 490-B and BF 490-C (conjugated metabolites of BF 490-2 and BF 490-9, respectively) were fortified with 0.3-0.5 ppm level. The exact fortification level of BF 490-B and BF 490-C were calculated from the measured radioactivity in the spiked sample and specific activity of the standard. All fortification samples were analyzed by BASF Method D9611. Recoveries for the analyses averaged 87 ± 11 (n = 11) for BAS 490 F, 90 ± 7 (n = 11) for BF 490-2, 85 ± 6 (n=11) for BF 490-9, 127 ± 0 (n = 2) for BF 490-B, and 115 ± 1 (n = 2) for BF 490-B. A summary of results are given in Table I and the individual results are given in Table III-VI. A summary of study dates is given in Table II.

Quantitation of all samples was achieved using calibration curves calculated by linear regression of standard data of multiple levels. The standard data for each analysis set are summarized in Table VIII.

8.2 Quantitation Limit

The limit of quantitation (LOQ) for Method D9611 for pecan nutmeat is 0.05 ppm for each analyte. These are the lowest levels for which the method has been tested and good fortification recovery data obtained. For pecan nutmeat matrices the control samaples were relatively clean and good recoveries were obtained from fortified samples.

8.3 Ruggedness Testing

Two analysts executed 3 sample sets for pecan. Two sets contained a control and duplicate analyses of control fortifed with at 0.05, 0.25 and 1.0 ppm levels of BAS 490 F, BF 490-2 and BF 490-9. One set contained a control and duplicate analysis of BF 490-B and BF 490-C (conjugated metabolites of BF 490-2 and BF 490-9 respectively).

The method has been used successfully to analyze fortified pecan samples. The recoveries and standard deviations for BAS 490 F, BF 490-2, BF 490-9, BF 490-B and BF 490-C in pecan are 87 ± 11 (n = 11), 90 ± 7 (n = 11), 85 ± 6 (n = 11), 127 ± 0 (n = 2), and 115 ± 1 (n = 2), respectively.

8.4 Limitations

BASF method No. D9611 was developed for the determination of BAS 490 F, and the glucoside conjugated metabolites BF 490-B and BF 490-C as BF 490-2 and BF 490-9, respectively in pecan. With BASF method D9611 the limit of quantitation was 0.05 ppm per analyte. This is the level where fortified compounds could be consistently recovered. The limit of detection based on the lowest standard injected, was about one-half of this level, at 0.025 ppm per analyte.

8.5 Interference and Potential Problems

To maintain the level of ruggedness and reliability of this method, it is important to perform all steps of the method protocol exactly as described.

Previous studies (Reference 8) showed that enzyme hydrolysis is the only effective means of releasing the conjugates to their relevent free metabolites. The enzymes are stable and have performed very consistently (References 9- 12)

It is essential to test and verify the elution profiles and retention times of BF 490-1, BF 490-2 and BF 490-9 on all column types used for HPLC under "native" conditions, i.e. in the presence of extract matrix before a sample series is started. Column testing with calibration standards can be time-optimized by first injecting a high concentration of each of the analytes dissolved separately in appropriately pre-prepared extracts from test samples. Then a standard prepared with all 3 analytes in a pre-prepared extract can be injected for correct window setting. The NH_2-phases of the analytical HPLC column from different suppliers may vary with respect to their NH_2-activity. Therefore, to ensure stable peak retention times of the three metabolites it might be required to change the concentration of formic acid in the HPLC-NH_2-column elution buffer (VII) to a value different than specified in this method, especially when new columns are used. For alterations of the elution protocol also refer to chapter 5.3.7 of this method.

So far no interferences of the detection systems for all three analytes with matrix compounds or other crop protectants were observed.

A specificity study has been conducted to test for interferences of other pesticides registered on pecan . No interferences have been found. The pesticides are listed in Table V and the study is described in Reference 7. Reference 7 describes Method 350/3 which has similar HPLC performance conditions as Method D9611.

9.0 Safety and Health Considerations

9.1 General

Use personal protective equipment such as lab coats, safety glasses and gloves (nitrile/latex gloves are recommended) when performing the operations described in this method. Conduct all filtrations, nitrogen-stream evaporations and SPE procedures in a well-ventilated hood. Guard vacuum equipment, such as rotovaps, to minimize the possibility of injury caused by flying broken glass. Dispose of hazardous wastes in an environmentally acceptable manner, in compliance with applicable laws and regulations.

9.2 Solvents and Reagents

Review the Material Safety Data Sheets (MSDSs) for all solvents and reagents used in this method.

10.0 Conclusions

This study has shown that Analytical Method Number D9601 is suitable for measuring residues of BAS 490 F and its metabolites down to a quantitative limit of 0.05 ppm. The average percent recovery for all analyses is 91 ± 13 (n = 37). The analytical method number D9611 showed specificity for determination of BAS 490 F and its metabolites in the presence of other pesticides having tolerances in pecan.

Statistical treatment of the validation data included determination of an average and standard deviation. Generally, good recoveries were obtained for the fortified pecan at the 0.05, 0.25 and 1.0 ppm levels.

The raw data and final method pertaining to this study are maintained in the BASF Corporation Agricultural Products Center Archives.

11.0 References

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6. Mackenroth, C. and Krotzky, A. Determination of the Stability of BF 490-2, BF 490-6, BF 490-9 and BF 490-18 in HPLC Eluent. BASF Registration Document No. 94/11107 December 1994. Limburgerhof, Germany.

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8. Rabe, U. and Mackenroth, C. "Validation of BASF Analytical Method No. 350/3: Determination of BAS 490 F (Kresoxim-methyl) and Its Metabolites BF 490-2 and BF 490-9 (free and glycosilated forms) in Tomato, Red Pepper, Melon, Cucumber, Onion, Grapes and Process Fractions, Apple and Apple Process Fractions. BASF Agricultural Chemicals Method Number 350/3. September 1996. Limburgerhof, Germany. BASF Registration Document Number 96/10626. Limburgerhof, Germany.

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10. Wofford, T., Movassaghi, S. and Riley, M. Magnitude of BAS 490 02F Residues in Apple Process Fractions. BASF Registration Document Number 96/5118. September 26, 1996.

11. Movassaghi, S., Jackson, S., and Riley, M. Magnitude of BAS 490 02F Residues in Grapes. BASF Registration Document Number 96/5219. October 25,1996.

12. Movassaghi, S., Wofford, T., and Riley, M. Magnitude of BAS 490 02F Residues in Grape Process Fractions. BASF Registration Document Number 96/5138. September 26,1996.

12.0 Signatures

We, the undersigned, hereby declare that this study was performed under our supervision according to the procedure described herein, and that this report provides a true and accurate record of the results obtained.

Author/Study Director:

Sherry Movassaghi — 6/2/97

Sherry Movassaghi
BASF Corporation

Study Completion Date

Author:

Jesse B. Thornton — 6/2/97

Jesse B. Thornton

Date

Approved by:

Laura Sears — 6/2/97

Laura Sears
Technical Center Leader

Date

Signatures for Amended Report

Amended By:

Sherry Movassaghi — 6/24/97

Approved By:

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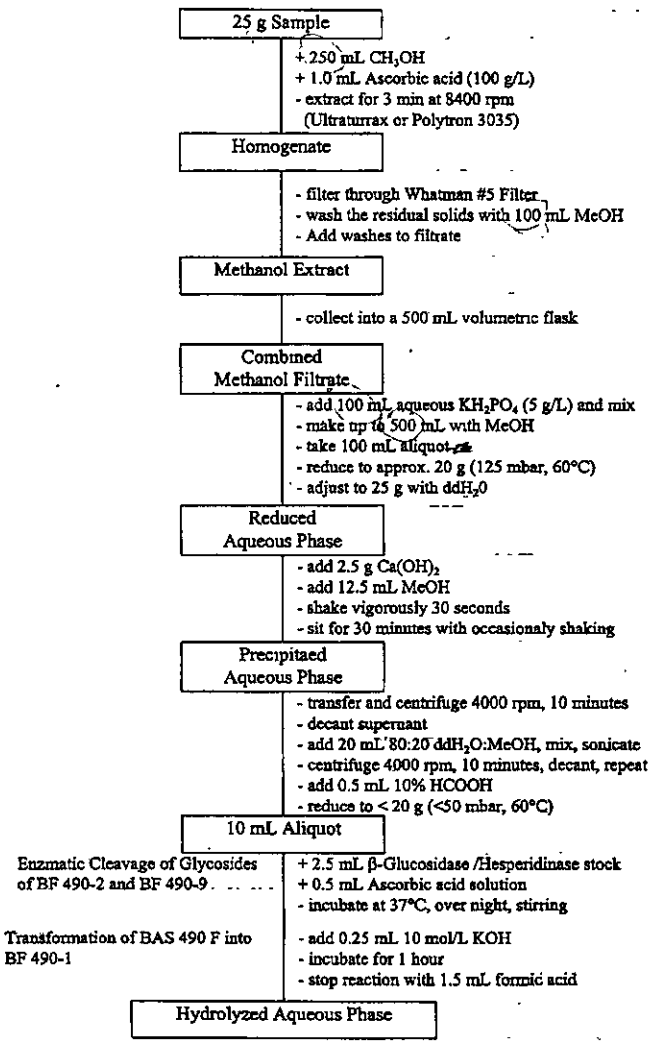
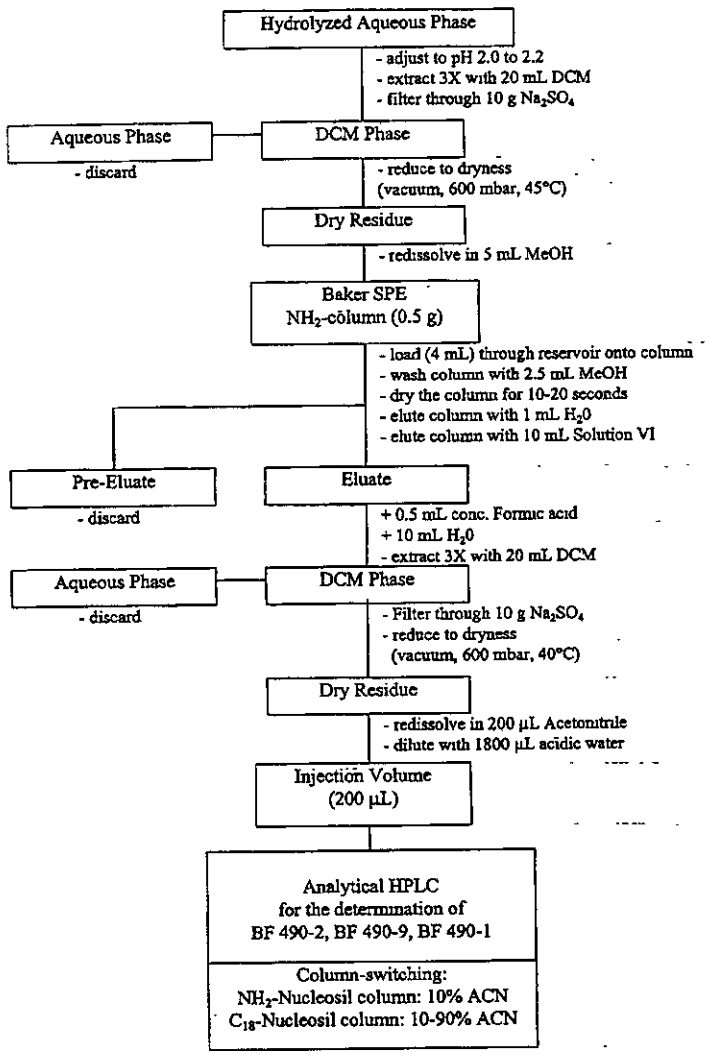


Figure 1. Flow Chart of Method D9611



1. **Flow Chart of Method D9611** (continued)

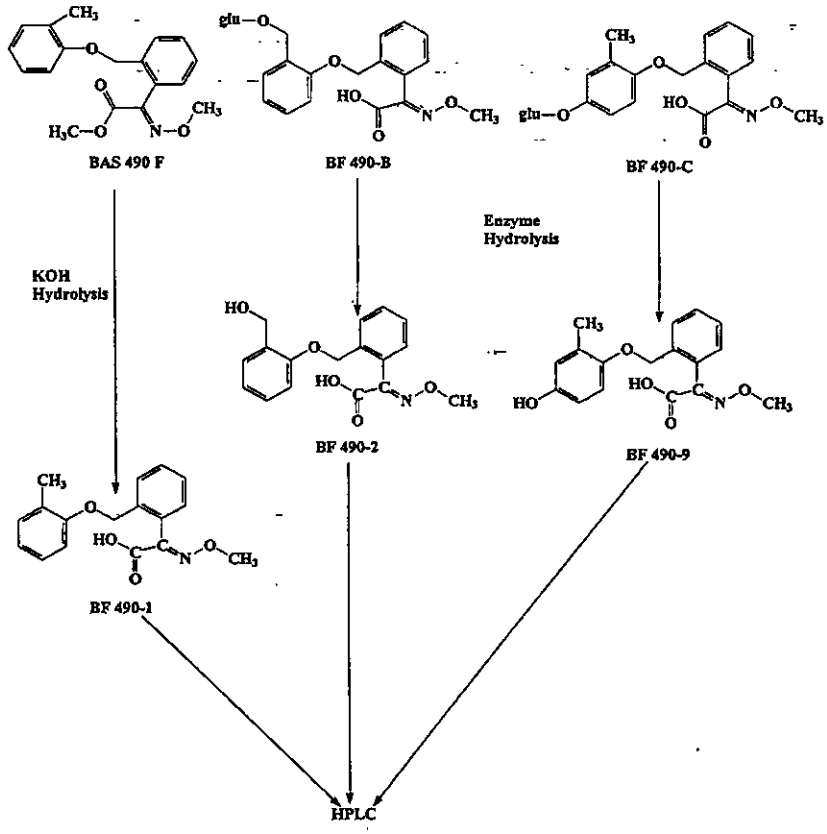


Figure 2. **Structures of BAS 490 F, its Conjugated Metabolites, and Final Analytes of BASF Analytical Method Number D9611**

Pecan control sample number 96178-0001 (run sequence no. 5) from Master Sheet 96163-03 fortified with 0.05 ppm BAS 490 F, BF 490-2 and BF 490-9. Data taken from Tables III and IV.

Each analyte concentration is interpolated from standard curve:

Standard Curve: BAS 490F Metabolite (ng) = $\frac{\text{Peak Height - Intercept}}{\text{Slope}}$

	BF 490-1	BF 490-2	BF 490-9
Peak Height (μV):	583	699	446
Slope:	3.70250E+1	3.64206E+1	3.24813E+1
Intercept:	-8.50005	-2.260883E+1	-2 849085E+1

BF 490-1 (ng) = $\frac{583 - -8.5005}{37.0250}$ = 15.976 ng

BF 490-2 (ng) = $\frac{699 - -22.60883}{36.4206}$ = 19.813 ng

BF 490-9 (ng) = $\frac{446 - -28.49085}{32.4813}$ = 14.608 ng

Residue (ppm) = $\frac{V_E \times W_A}{G \times V_I \times A}$

V_E = final volume (mL) after all dilution steps
W_A = amount of analyte (ng) interpolated from calibration curve
G = weight (g) of original sample extracted
V_I = injection volume (μL) taken from V_E
A = aliquot %, as a decimal, of original sample taken during processing

The following values were used to calculate Residue (ppm) for this sample:

V_E = 1.0 mL
W_A = BF 490-1 = 15.976 ng; BF 490-2 = 19.813 ng; BF 490-9 = 14.608 ng
G = 25 g
V_I = 200 μL
A = 0.08

BF 490-1 Residue (ppm) = $\frac{1.0 \text{ mL} \times 15.976 \text{ ng}}{25 \text{ g} \times 200 \text{ μL} \times 0.08}$ = 0.040 ppm

BF 490-2 Residue (ppm) = $\frac{1.0 \text{ mL} \times 19.813 \text{ ng}}{25 \text{ g} \times 200 \text{ μL} \times 0.08}$ = 0.050 ppm

BF 490-9 Residue (ppm) = $\frac{1.0 \text{ mL} \times 14.608 \text{ ng}}{25 \text{ g} \times 200 \text{ μL} \times 0.08}$ = 0.037 ppm

% Recovery = $\frac{\text{Residue in Fortified Sample (ppm) - Residue in Control (ppm)}}{\text{Amount Fortified (ppm)}} \times \text{MWCF}^1$

[1] MWCF is only needed for the conversion of BF 490-1 to BAS 490 F = 1.047.

BAS 490 F % Recovery = $\frac{0.040 \text{ ppm} - 0.00 \text{ ppm}}{0.05 \text{ ppm}} \times 1.047$ = 84 %

BF 490-2 % Recovery = $\frac{0.050 \text{ ppm} - 0.00 \text{ ppm}}{0.05 \text{ ppm}}$ = 100 %

BF 490-9 % Recovery = $\frac{0.037 \text{ ppm} - 0.00 \text{ ppm}}{0.05 \text{ ppm}}$ = 74 %

Use full computer/calculator precision in any intermediate calculations. Round only the final value.

Figure 3. Typical Recovery Calculation for HPLC Quantitation of BAS 490 F, BF 490-2 and BF 490-9

BF 490-B and BF 490-C fortified onto respective aliquots of pecan control sample number 96178-0001 from Master Sheet 96163-02. Data taken from Table III and IV.

Standard Curve: BAS 490 F Metabolite (ng) = $\frac{\text{Peak Height - Intercept}}{\text{Slope}}$

Each analyte concentration is interpolated from standard curve.

Residue (ppm) = $\frac{V_E \times W_A}{G \times V_I \times A}$

V_E = 2.0 mL = final volume (mL) after all dilution steps
G = 5.0 g = weight (g) of original sample extracted
V_I = 200 µL = injection volume (µL) taken from V_E
A = 0.36 = aliquot %, as a decimal, of original sample taken during processing
W_A = amount of analyte (ng) interpolated from calibration curve

% Recovery = $\frac{\text{Residue in Fortified Sample (ppm) - Residue in Control (ppm)}}{\text{Amount Fortified (ppm)}} \times \text{MWCF}^1$

[1] MWCF for converting BF 490-B and BF 490-C to their respective analytes, BF 490-2 and BF 490-9, equals 1.51.

0.512 ppm BF 490-B measured as BF 490-2, Run Sequence Number 5

Peak Height (µV): 2575
Slope: 3.42018E+1
Intercept: -7 393428E+1

BF 490-2 (ng) = $\frac{2575 - -73.93428}{34.2018}$ = 77.450 ng

BF 490-2 Residue (ppm) = $\frac{2.0 \text{ mL} \times 77.450 \text{ ng}}{5.0 \text{ g} \times 200 \text{ µL} \times 0.36}$ = 0.430 ppm

BF 490-B % Recovery = $\frac{0.430 \text{ ppm} - 0.00 \text{ ppm}}{0.512 \text{ ppm}} \times 1.51$ = 127 %

0.802 ppm BF 490-C measured as BF 490-9, Run Sequence Number 9

Peak Height (µV): 3342
Slope: 3.09723E+1
Intercept: -3.754248E+1

BF 490-9 (ng) = $\frac{3342 - -37.54248}{30.9723}$ = 109.11 ng

BF 490-9 Residue (ppm) = $\frac{2.0 \text{ mL} \times 109.11 \text{ ng}}{5.0 \text{ g} \times 200 \text{ µL} \times 0.36}$ = 0.606 ppm

BF 490-B % Recovery = $\frac{0.606 \text{ ppm} - 0.00 \text{ ppm}}{0.802 \text{ ppm}} \times 1.51$ = 114 %

Figure 4. Typical Recovery Calculation for HPLC Quantitation of BAS 490 F Conjugated Metabolites BF 490-B and BF 490-C

Table I. Summary of Recovery Data for Pecan

Compound Fortified	Fortification Level (ppm)	% Recovery Average ± Standard Deviation	Total Compound Average % Recovery ± Standard Deviation
BAS 490 F	0.05	96 ± 14 (n = 4)	87 ± 11 (n = 11)
	0.25	83 ± 5 (n = 4)	
	1.0	82 ± 6 (n = 3)	
BF 490-2	0.05	97 ± 7 (n = 4)	90 ± 7 (n = 11)
	0.25	87 ± 3 (n = 4)	
	1.0	86 ± 3 (n = 3)	
BF 490-9	0.05	87 ± 9 (n = 4)	85 ± 6 (n = 11)
	0.25	84 ± 6 (n = 4)	
	1.0	86 ± 4 (n = 3)	
BF 490-B	0.512	127 ± 0 (n = 2)	
BF 490-C	0.802	115 ± 1 (n = 2)	
Average % Recovery for all Analyses = 91 ± 13 (n = 37)			

See Table III for individual recovery results.

Table II. Summary of Study Dates

Residue Sample Number	Master Sheet Number (96163-)	Analyte	Extraction Date	Injection Date
96178-0001	02	BF 490-B BF 490-C	01-09-97	01-11-97
96178-0001	03	BAS 490 F BF 490-2 BF 490-9	01-14-97	01-15-97
96108-0001	04	BAS 490 F BF 490-2 BF 490-9	01-21-97	01-22-97

Table IIL Individual Recovery Data for Pecan Nutmeat

Fortified Level (ppm)[1]	Run Sequence Number[2]	Master Sheet Number (96163-)	Final Volume (mL)	Peak Height (µV)[3,4]	Net Residue (ppm)[4]	Recovery (%)[5,6]
BAS 490 F						
0.00	3	03	1.0	nd	< 0.05	—
0.00	3	04	1.0	nd	< 0.05	—
0.05	5	03	1.0	583	0.040	84
0.05	7	03	1.0	586	0.040	84
0.05	4	04	1.0	600	0.049	103
0.05	6	04	1.0	656	0.053	111
0.25	9	03	2.0	1319	0.179	75
0.25	10	03	2.0	1498	0.204	85
0.25	7	04	2.0	1422	0.208	87
0.25	9	04	2.0	1351	0.199	83
1.0	12	03	2.0	5815	0.786	82
1.0	13	03	2.0	6105	0.826	87
1.0	12	04	2.0	5260	0.722	76
BF 490-2						
0.00	3	03	1.0	nd	< 0.05	—
0.00	3	04	1.0	nd	< 0.05	—
0.05	5	03	1.0	699	0.050	100
0.05	7	03	1.0	652	0.046	92
0.05	4	04	1.0	565	0.045	90
0.05	6	04	1.0	661	0.052	104
0.25	9	03	2.0	1480	0.206	82
0.25	10	03	2.0	1593	0.222	89
0.25	7	04	2.0	1504	0.222	88
0.25	9	04	2.0	1513	0.223	89
1.0	12	03	2.0	6095	0.840	84
1.0	13	03	2.0	6447	0.888	89
1.0	12	04	2.0	5974	0.850	85
BF 490-9						
0.00	3	03	1.0	nd	< 0.05	—
0.00	3	04	1.0	nd	< 0.05	—
0.05	5	03	1.0	446	0.037	74
0.05	7	03	1.0	532	0.043	86
0.05	4	04	1.0	497	0.045	90
0.05	6	04	1.0	530	0.048	96
0.25	9	03	2.0	1215	0.191	76
0.25	10	03	2.0	1338	0.210	84
0.25	7	04	2.0	1366	0.226	90
0.25	9	04	2.0	1300	0.216	86
1.0	12	03	2.0	5625	0.870	87
1.0	13	03	2.0	5760	0.891	89
1.0	12	04	2.0	5103	0.811	81

Table III. Individual Recovery Data for Pecan Nutmeat (continued)

Fortified Level (ppm)[1]	Run Sequence Number[2]	Master Sheet Number (96163-)	Final Volume (mL)	Peak Height (μV)[3,4]	Net Residue (ppm)[4]	Recovery (%)[5,6]
BF 490-B						
0.00	3	2	2.0	nd	<0.05	---
0.512	5	2	2.0	2575	0.430	127
0.512	7	2	2.0	2588	0.432	127
BF 490-C						
0.00	3	2	2.0	nd	<0.05	---
0.802	9	2	2.0	3342	0.606	114
0.802	11	2	2.0	3421	0.620	116

ND = Not Detected

The following data were constant on all samples fortified with BAS 490F, BF 490-2 and BF 490-9:
Sample Weight = 25.0 g Aliquot % = 0.08 Injection Volume = 200 μL

The following data were constant on all samples fortified with BF 490-B and BF 490-9:
Sample Weight = 5.0 g Aliquot % = 0.36 Injection Volume = 200 μL

1 Fortifications were added prior to extraction and were run concurrently with control sample.
2 Run Sequence Numbers were assigned to distinguish between separate analyses of the same sample number within a sample set.
3 If no signal was detected 'ND' is listed in the table.
4 Peak heights and net residue of BAS 490F, BF 490-B and BF 490-C are shown as BF 490-1, BF 490-2 and BF 490-9, respectively.
5 See Figure 3 for an example calculation of this value for BAS 490F, BF 490-2 and BF 490-9.
6 See Figure 4 for an example calculation of this value for BF 490-B and BF 490-C.

Table IV. Summary of Standard Data

Master Sheet Number (96163-)[1]	Analyte	Peak Height (μV)				Calibration Curve[2] Data	
		10 ng	25 ng	100 ng	250 ng	Slope	Intercept
02	BF 490-2	252	749	3420	8471	3.42018E+1	-7.393428E+1
		291	723	3397	8441		
	BF 490-9	271	680	3087	7692	3.09723E+1	-3.754248E+1
		325	703	3084	7706		
03	BF 490-1	354	852	3796	9341	3.70250E+1	-8.50005
		359	876	3768	9096		
	BF 490-2	354	829	3716	9151	3.64206E+1	-2.260883E+1
		300	811	3771	8930		
	BF 490-9	276	720	3339	8192	3.24813E+1	-2.849085E+1
		277	721	3353	7905		
04	BF 490-1	185	761	3596	9260	3.73479E+1	-1.346661E+2
		340	871	3477	9190		
	BF 490-2	303	797	3521	8852	3.55506E+1	-7.240327E+1
		337	824	3344	8818		
	BF 490-9	146	721	3125	7932	3.19470E+1	-7.918050E+1
		353	744	3036	7908		

1 Master Sheet numbers consist of the BASF study number (96163) followed by a sequential analysis set number The Extraction and Injection dates are entered on the Master Sheets and in the Summary of Study Dates Table (see Table II).

2 The standard curves were constructed using the following equation:

$$\text{Analyte (ng)} = \frac{\text{Peak Height - Intercept}}{\text{Slope}}$$

APPENDIX A

Protocol Changes

rotocol Changes

One change was issued to the protcol during the analysis of the study.

Change Number 1added a pecan control sample RCN 96104-0001 from pecan RAC study 96044 because there was not enought control sample to spike the fortification samples.

This change to the protocol had no effect on the final results of this study.

APPENDIX B

Typical Raw Data and Chromatograms

Figure 1. **Typical HPLC parameters**

HPLC
INSTRUMENT PARAMETERS

Date: 6/15/97 Initials: JT

Study No: 96163 Master Sheet No: 96163 - 03

BASF Instrument No :1233 LC Model: HP 1090 L/M
Pump/Controller No.:580 Data System No : 676
Detector No.: 580 Autosample No :580 Inj. Vol.: 200 µl

Columns: Column Switching
Column I: Amino Column: Nucleosil NH_2 Serial No.: 160994 Column ID: 4.0 mm
Particle Shape/Size: Spher/5 u Pore Size: 100°A Length: 100 mm

Column II: Analytical Column: Nucleosil C_{18} Serial No.: 160995 Column ID: 4.6 mm
Particle Shape/Size: Spher/5 u Pore Size: 100°A Length: 250 mm

Columns Temp.: Room Temp.
Column Switching Times: Checked before each run. Typical switching times are listed in the following table.

Time (min)	Switch
5.0	open
6.5	closed
7.3	open
9.0	closed

Mobile Phase:
Mobile Phase: Amino Column: Isocratic
90:10:1.0 (v/v/v) ddH_2O:Acetonitrile:Formic Acid

C_{18} Column: Gradient
A: 90:10:0.5 (v/v/v) ddH_2O:Acetonitrile:Formic Acid
B: 10:90:0.5 (v/v/v) ddH_2O:Acetonitrile:Formic Acid

Typical Gradient and Stop Flow Times are listed in the following Table:

Time (min)	% B	Flow (mL/min)
0.0	0.0	0.7
5.3	0.0	0.7
5.4	0.0	0.0
8.6	0.0	0.0
8.7	0.0	0.7
32.0	70.0	0.7
34.0	100.0	0.7
37.0	100.0	0.7
38.0	0.0	0.7

Flow Amino Column: 0.7 mL/min
Flow C_{18} Column: 0.7 mL/min

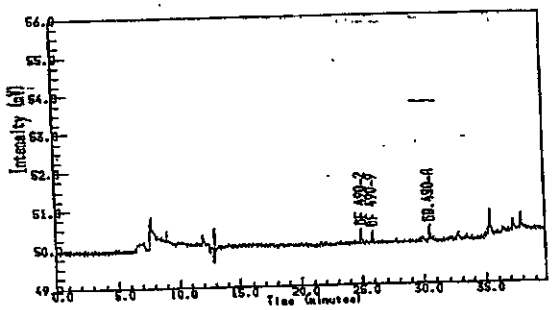
Detector:

Detector Wavelength: 270 nm
Detector Type: UV

Figure 2. Typical chromatogram of a 10 ng standard of BF 490-1, BF 490-2 and BF 490-9 from Master Sheet Number 96163-03 (run sequence number 1). See Table IV.

[96163] 25 9616303.1.1
Reported on 16-JAN-1997 at 09:10
Modified on 16-JAN-1997 at 09:08

Acquired on 15-JAN-1997 at 19:34



BASF CORP. - VAX MULTICHROM

Analyst Name : JESSE
Lims Id :
Comment :
Method Title : Analysis of BAS 490...F Metabolites
Sample Name : 10 ng STD
Sample Id :
Sample Type : Standard Amount=1.00000
Bottle No : 1

PEAK INFORMATION

RT mins	Hght uV	Area uVs	ppm	Peak name
24.907	354	2964	10.353	BF 490-2
25.849	276	1735	9.389	BF 490-9
30.453	354	2269	9.788	BF 490-1
Totals				
Unknowns	354	2269	N/A	
	985	6969	29.531	
	1339	9238	29.531	

Figure 3. Typical chromatogram of a 25 ng standard of BF 490-1, BF 490-2 and BF 490-9 from Master Sheet Number 96163-03 (run sequence number 2). See Table IV.

[96163] 25 9616303.2.1
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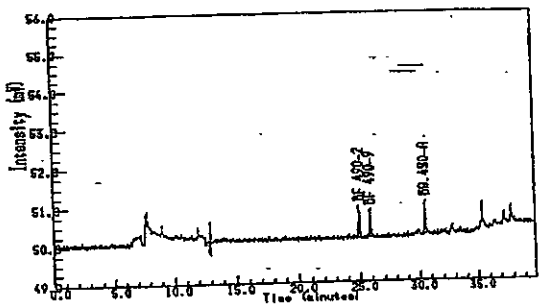
Modified on 16-JAN-1997 at 09:08

Acquired on 15-JAN-1997 at 20:25



BASF CORP. - VAX MULTICHROM

Analyst Name : JESSB
Lims Id :
Comment :
Method Title : Analysis of BAS 490...F Metabolites
Sample Name : 25 ng STD
Sample Id :
Sample Type : Standard Amount=1.00000
Bottle No : 1

PEAK INFORMATION

RT mins	Hght uV	Area uVs	ppm	Peak name
24.907	829	6097	23.388	BF 490-2
25.849	720	5095	23.040	BF 490-9
30.453	852	6726	23.247	BF 490-A
Totals				
Unknowns	852	6726	N/A	
	2401	17917	69.676	
	3254	24643	69.676	

Figure 4. Typical chromatogram of a 100 ng standard of BF 490-1, BF 490-2 and BF 490-9 from Master Sheet Number 96163-03 (run sequence number 4). See Table IV.

[96163] 2S 9516303.4,1
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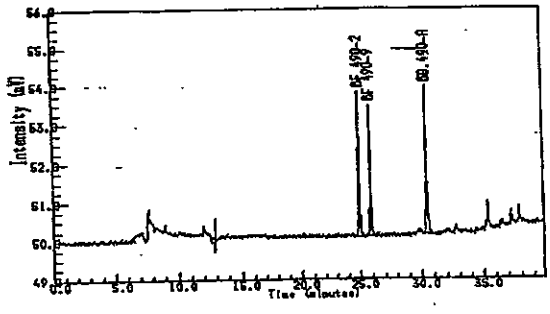
Acquired on 15-JAN-1997 at 22:07



BASF CORP. - VAX MULTICHROM

Analyst Name : JESSE
Lims Id :
Comment :
Method Title : Analysis of BAS 490...F Metabolites
Sample Name : 100 ng STD
Sample Id :
Sample Type : Standard Amount=0.00000
Bottle No : 1

PEAK INFORMATION

RT mins	Hght uV	Area uVs	ppm	Peak name
24.898	3716	27388	102.643	BF 490-2
25.831	3339	23529	103.661	BF 490-9
30.444	3796	30158	102.754	BF 490-A (BF490-1)

Totals			
Unknowns	3796	30188	N/A
	10850	81074	309.057
	14646	111232	309.057

Figure 5. Typical chromatogram of a 250 ng standard of BF 490-1, BF 490-2 and BF 490-9 from Master Sheet Number 96163-03 (run sequence number 6). See Table IV.

[96163] 25 9616303,6,1
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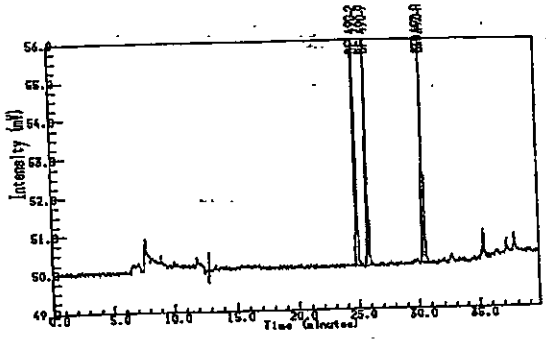
Acquired on 15-JAN-1997 at 23:50



BASF CORP. - VAX MULTICHROM

Analyst Name : JESSE
Lims Id :
Comment :
Method Title : Analysis of BAS 490...F Metabolites
Sample Name : 250 ng STD
Sample Id :
Sample Type : Standard Amount=1.00000
Bottle No : 1

PEAK INFORMATION

RT mins	Hght uV	Area uVs	ppm	Peak name
24.880	9151	66933	251.867	BF 490-2
25.813	8192	59909	253.079	BF 490-9
30.418	9341	73873	252.510	BF 490-A (BF490-1)
Totals				
Unknowns	9341	73873	N/A	
	26683	200715	757.456	
	36024	274589	757.456	

Figure 6. Typical standard curve for 10, 25, 100 and 250 ng amounts of BF 490-1 from Master Sheet 96163-03. See Table IV.

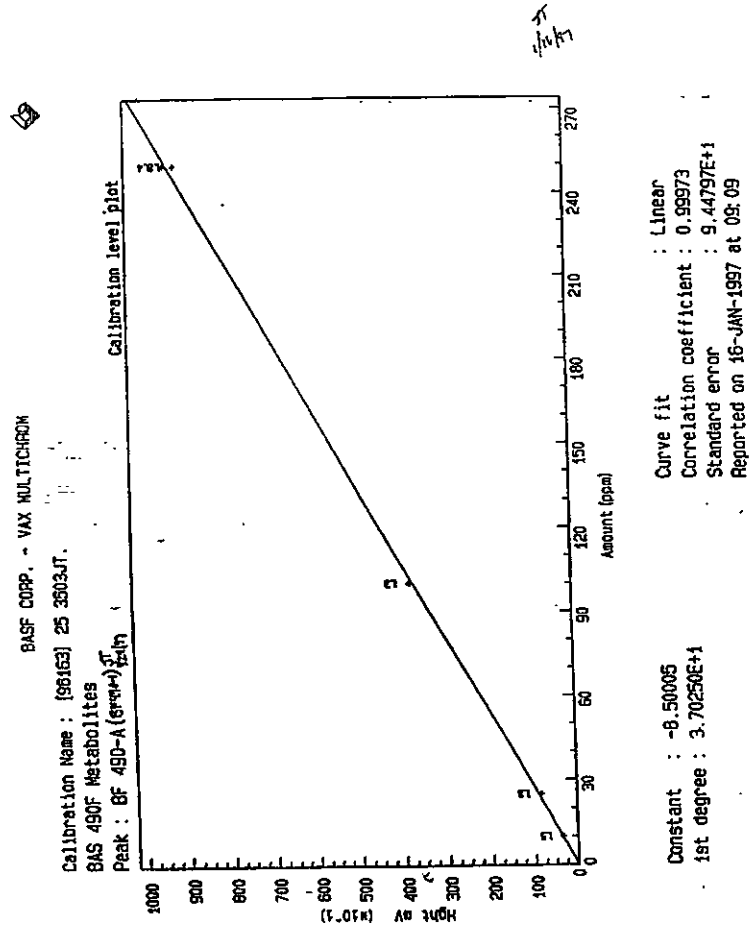


Figure 7. **Typical standard curve for 10, 25, 100 and 250 ng amounts of BF 490-2 from Master Sheet 96163-03. See Table IV.**

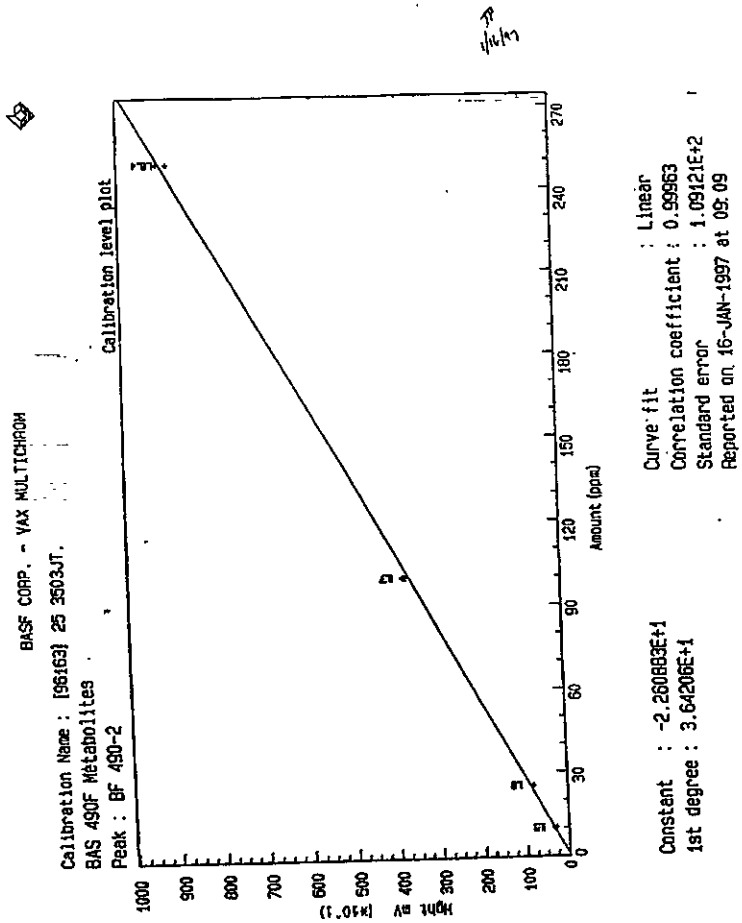


Figure 8. Typical standard curve for 10, 25, 100 and 250 ng amounts of BF 490-9 from Master Sheet 96163-03. See Table IV.

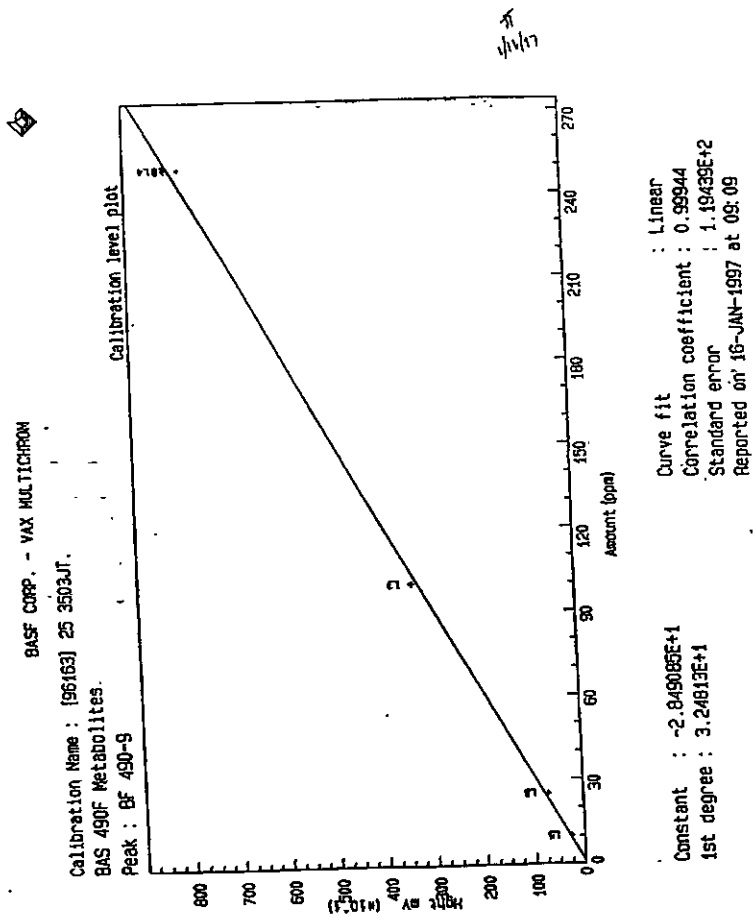


Figure 9. Chromatogram of a control pecan sample. Sample number 96178-0001 (run sequence number 3). Master Sheet 96163-03. See Table III.

[96163] 25 9616303,3,1
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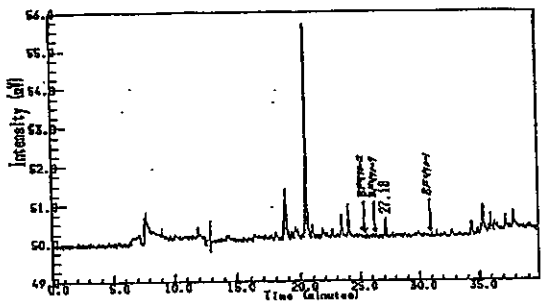
Modified on 15-JAN-1997 at 09:08

Acquired on 15-JAN-1997 at 21:16



BASF CORP. - VAX MULTICHROM

Analyst Name : JESSE
Lims Id :
Comment :
Method Title : Analysis of BAS 490...F Metabolites
Sample Name : control, pecan
Sample Id : 96178-0001
Sample Type : Control Amount=25.00000
Bottle No : 1

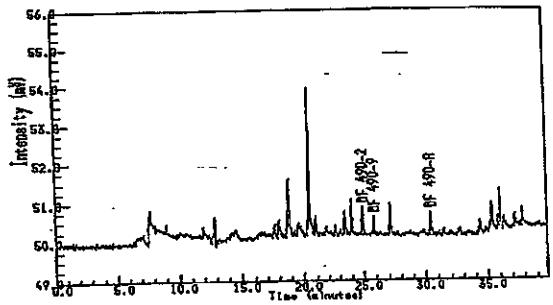
PEAK INFORMATION

RT mins	Hght uV	Area uVs	ppm	Peak name
Totals				
Unknowns	477	3628	N/A	
	0	0	0.000	
	477	3628	0.000	

Figure 10. Chromatogram of a control pecan sample fortified with 0.05 ppm (the quantitation limit) of BAS 490 F, BF 490-2 and BF 490-9. Sample number 96178-0001 (run sequence number 5). Master Sheet 96163-03. See Table III.

[96163] 25 9616303.5,1
Reported on 16-JAN-1997 at 09:10
Modified on 16-JAN-1997 at 09:08

Acquired on 15-JAN-1997 at 22:58



BASF CORP. - VAX MULTICHROM

Analyst Name : JESSE
Lims Id :
Comment :
Method Title : Analysis of BAS 490...F Metabolites
Sample Name : Proc Fort I
Sample Id : 96178-0001
Sample Type : Recovery Amount=25.00000
Bottle No : 1

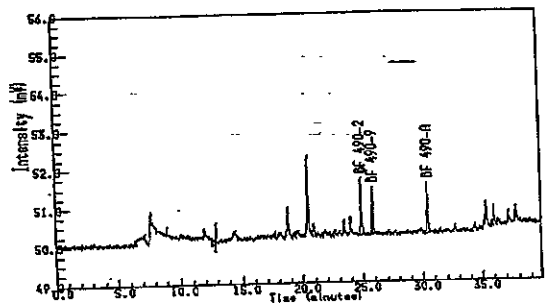
PEAK INFORMATION

RT mins	Hght uV	Area uVs	ppm	Peak name
24.907	699	5486	0.050	BF 490-2
25.831	446	3292	0.037	BF 490-9
30.427	583	5273	0.040	BF 490-A (BF490-8)
Totals				
Unknowns	0	0	N/A	
	1728	14051	0.126	
	1728	14051	0.126	

Figure 11. Chromatogram of a control pecan sample fortified with 0.25 ppm of BAS 490 F, BF 490-2 and BF 490-9. Sample number 96178-0001 (run sequence number 9). Master Sheet 96163-03. See Table III.

[96163] 25 9616303.9.1
Reported on 16-JAN-1997 at 09:11
Modified on 16-JAN-1997 at 09:08

Acquired on 16-JAN-1997 at 02:23



BASF CORP. - VAX MULTICHROM

Analyst Name : JESSE
Lims Id :
Comment :
Method Title : Analysis of BAS 490...F Metabolites
Sample Name : Proc Fort III
Sample Id : 96178-0001
Sample Type : Recovery Amount=25.00000
Bottle No : 1

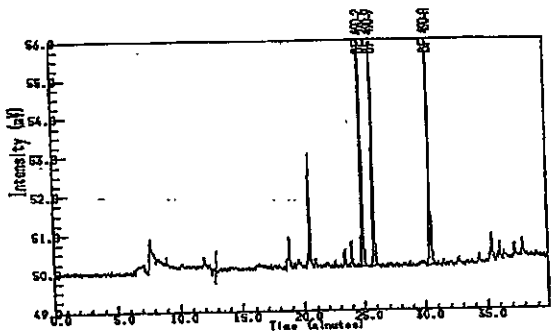
PEAK INFORMATION

RT mins	Hght uV	Area uVs	ppm	Peak name
24.871	1480	11406	0.206	BF 490-2
25.796	1215	8863	0.191	BF 490-9
30.409	1319	10664	0.179	BF 490-A (free acid)
Totals				
Unknowns	0	0	N/A	
	4014	30933	0.577	
	4014	30933	0.577	

Figure 12. Chromatogram of a control pecan sample fortified with 1.0 ppm of BAS 490 F, BF 490-2 and BF 490-9. Sample number 96178-0001 (run sequence number 13). Master Sheet 96163-03. See Table III.

[96163] 25 9616303,13,1
Reported on 16-JAN-1997 at 09:11
Modified on 16-JAN-1997 at 09:08

Acquired on 16-JAN-1997 at 05:47..



BASF CORP. - VAX MULTICHROM

Analyst Name : JESSE
Lims Id :
Comment :
Method Title : Analysis of BAS 490...F Metabolites
Sample Name : Proc Fort VI
Sample Id : 96178-0001
Sample Type : Recovery Amount=25.00000
Bottle No : 1

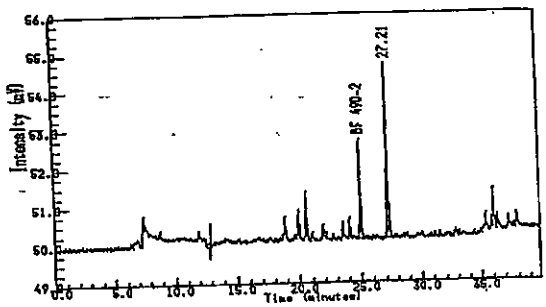
PEAK INFORMATION

RT mins	Hght uV	Area uVs	ppm	Peak name
24.880	6447	46676	0.686	BF 490-2
25.813	5760	41443	0.691	BF 490-9
30.427	6105	48379	0.826	BF 490-A
Totals				
Unknowns	0	0	N/A	
	18312	136498	2.605	
	18312	136498	2.605	

Figure 13. Chromatogram of a control pecan sample fortified with 0.512 ppm of BF 490-B. Sample number 96178-0001 (run sequence number 5). Master Sheet 96163-02. See Table III.

[96163] 25 9616302.5,1
Reported on 13-JAN-1997 at 08:17
Modified on 13-JAN-1997 at 08:15

Acquired on 11-JAN-1997 at 15:31



BASF CORP. - VAX MULTICHROM

Analyst Name : JESSE
Lims Id :
Comment :
Method Title : Analysis of BAS 490...F Metabolites
Sample Name : Proc Fort I
Sample Id : 96178-0001
Sample Type : Recovery Amount=5.00000
Bottle No : 1

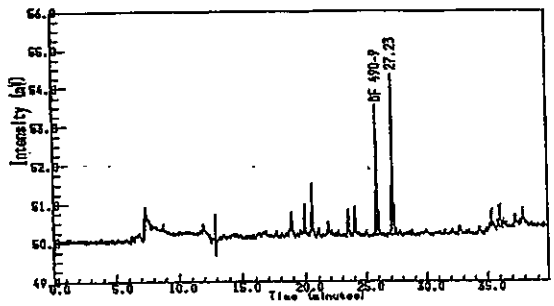
PEAK INFORMATION

RT mins	Hght uV	Area uVs	ppm	Peak name
24.942	2575	19453	0.430	BF 490-2
Totals				
Unknowns	4572	36190	N/A	
	2575	19453	0.430	
	7147	55643	0.430	

Figure 14. Chromatogram of a control pecan sample fortified with 0.802 ppm of BF 490-C. Sample number 96178-0001 (run sequence number 9). Master Sheet 96163-02. See Table III.

[96163] 25 9616302,9,1
Reported on 13-JAN-1997 at 08:17
Modified on 13-JAN-1997 at 08:15

Acquired on 11-JAN-1997 at 18:58



BASF CORP. - VAX/MULTICHROM

Analyst Name : JESSE
Lims Id :
Comment :
Method Title : Analysis of BAS 490...F Metabolites
Sample Name : Proc Port III
Sample Id : 96178-0001
Sample Type : Recovery Amount=5.00000
Bottle No : 1

PEAK INFORMATION

RT mins	Hght uV	Area uVs	ppm	Peak name
25.902	3342	25056	0.606	BF 490-9
Totals				
Unknowns	4162	32798	N/A	
	3342	25056	0.606	
	7503	57854	0.606	

ATTACHMENTS

Attachment 1:

Sigma Quality Control Test Procedure for Hesperidinase (EC 3.2.1.21)

Principle: Hesperidin + H_2O $\xrightarrow{\text{Hesperidinase}}$ D-Glucose + Rhamnose

Conditions: T = 40 °C, pH = 3.8, A_{540nm}, Light path = 1 cm

Method: Colorimetric

Reagents:

A. 200 mM Sodium Phosphate Solution
(Prepare 100 mL in deionized water using Sodium Phosphate, Monobasic, Prod. No. S-0751. Adjust to pH 7.6 at 25 °C with 1 M NaOH.)

B. 100 mM Citric Acid Solution
(Prepare 100 mL in deionized water using Citric Acid, Monohydrate, Prod. No. C-7129.)

C. McIlvaine Buffer, pH 3.8 at 40 °C
(Prepare 100 mL using Reagent A. Adjust to pH 3.8 at 40 °C with Reagent B.)

D. 100 mM Sodium Hydroxide Solution (NaOH)
(Prepare 100 liter in deionized water using Sodium Hydroxide, Prod. No. S-5881.)

E. 2.1 mM Hesperidin Substrate Solution
(Prepare by dissolving 120 mg of Hesperidin, Prod. No. H-5254, in 10 mL of Reagent D. Add 40 mL of Reagent C and adjust to pH 3.8 with 1 M HCl.)

F. Hesperidinase Enzyme Solution
(Immediately before use, prepare a solution containing 0.3 - 0.6 units/mL of Hesperidinase in cold deionized water.)

Reagents: (continued)

G: Copper Solution
The following components are made separately:

1. 2.53 M Sodium Sulfate, 453 mM Sodium Carbonate, and 85 mM Sodium Potassium Tartrate Solution (Prepare 500 mL in deionized water using Sodium Sulfate, Prod. No. S-9627, Sodium Carbonate, Prod. No. S-2127, and Sodium Potassium Tartrate, Tetrahydrate, Prod. No. S-2377. PREPARE FRESH.)

2. 481 mM Cupric Sulfate Solution (Prepare 100 mL in deionized water using Cupric Sulfate, Pentahydrate, Prod. No. C-7631. PREPARE FRESH.)

Place component 1 into a suitable container and mix with a magnetic stirrer. Slowly add component 2 (Cupric Sulfate). After these components are combined, add 16 grams of Sodium Bicarbonate, Prod. No. S-8875. Add enough deionized water to make the total volume 1 liter. Store in amber bottle.

H. Arsenomolybdate Solution (Ar-Mol)
The following components are made separately:

1. 135 mM Ammonium Molybdate Solution (Prepare 300 mL in deionized water using Molybdic Acid, Ammonium Salt, Tetrahydrate, Prod. No. M-0878. PREPARE FRESH.)

2. 64 mM Arsenic Acid Solution (Prepare 300 mL in deionized water using Arsenic Acid, Sodium Salt, Heptahydrate, Prod. No. A-6756. PREPARE FRESH.)

Place component 1 into a suitable container and mix with a magnetic stirrer. Slowly add 12 mL of Concentrated Sulfuric Acid. Then add component 2 (Arsenic Acid). After these components are combined, add enough deionized water to make the total volume 1 liter. Incubate at

37°C for 48 - 72 hours. Store in amber bottle.

I. 1 mg/mL Sigma Glucose Standard Solution (Use Stock No. 635-100)

Procedure: Pipette (in milliliters) the following into reagents into suitable test tubes:

	Test	Blank
Reagent E (Hesperidin)	4.00	4.00
Equilibrate to 40°C. Then add:		
Deionized Water	-----	1.00
Reagent F (Enzyme)	1.00	
Mix and incubate at 40°C for exactly 30 minutes. Then add:		
Reagent G (Copper)	5.00	5.00

Mix well.

Pipette (in milliliters) the following reagents into suitable containers:

	Test	Test Blank	Std 1	Std 2	Std 3	Std Blank
Test Solution	2.00	-----	-----	-----	-----	-----
Test Blank Soln.	------	2.00	-----	-----	-----	-----
Deionized Water	------	------	0.85	0.90	0.95	1.00
Reagent I (Glucose)	------	------	0.15	0.10	0.05	-----
Reagent G (Copper)	------	-----	1.00	1.00	1.00	1.00

Immediately mix. Place a marble over the top of the tube and transfer the tubes to a boiling water bath. Incubate for 10 minutes. Remove from boiling water bath and allow to cool to room temperature. Then add:

Reagent H (Are-Mol) 1.00 1.00 1.00 1.00 1.00 1.00

Shake or vortex until foaming stops and any precipitate present is dissolved. Then add:

Dionized Water 10.00 10.00 10.00 10.00 10.00 10.00

Mix and transfer suitable cuvettes. Obtain the A_{540nm} for Test, Blank and Standards, using a suitable spectrophotometer.

Calculations:

$$A_{540nm}\ \text{Std} = \frac{A_{540nm}\ \text{Std} - A_{540nm}\ \text{Std Blank}}{(\text{mg Glucose/Std})\ (5.55)}$$

$$\_A_{540nm}\ \text{Std/mmole} = \frac{(A_{540nm}\ \text{Std 1}) + (A_{540nm}\ \text{Std 2}) + (A_{540nm}\ \text{Std 3})}{3}$$

$$\text{Units/mg enzyme} = \frac{A_{540nm}\ \text{Test} - A_{540\ nm}\ \text{Test Blank})\ (5)}{(30)\ (\text{mg enzyme/RM})\ (\_A_{540nm}\ \text{Std/mmole})}$$

5.55	=	micromole per milligram of glucose
3	=	Average of the standards
5	=	Volume of Reaction Mix
30	=	Time of Assay in minutes (Unit Definition)
RM	=	Reaction Mix (Volume = 5.0 mL)

Unit definition:

One unit will liberate 1.0 µmole of reducing sugar (as glucose) from hesperidin per minute at pH 3.8 at 40 °C.

Final assay concentrations:

In a 5 mL reaction mix, the final concentrations are 1.68 mM hesperidin and 0.3 - 0.6 Units hesperidinase. (Note: Concentration of the buffers are not exact due to the method of preparation of the McIlvaine's Buffer.)

Notes:

1. The Sodium Bicarbonate may need to be dissolved in water before adding to the solution.

2. If a precipitate forms, it should be removed by filtration prior to use.

3. Reagent is light sensitive. Avoid exposure to direct sunlight.

4. Caution, add carefully. The addition could cause the material to heat rapidly.

5. All product and stock numbers, unless otherwise indicated, are Sigma product and stock numbers.

Attachment 2:

Qualitative Assay of Hesperidinase Activity by TLC

Purpose: To determine activity of Hesperidinase by TLC using the substrate Hesperidin and a normal phase solvent system.

Principal: The enzyme Hesperidinase will liberate 1.0 µmole of reducing sugar, as glucose, from hesperidin per minute at pH 3.8 at 40°C.

Test Substances:

	Hesperidinase Enzyme	Sigma Cat-No. H-8137
or	Hesperidinase Enzyme	ICN Cat.-No. 157339

Equipment Used:

Eppendorf Pipettors	100 and 1000 µL sizes
Drummond Micropipet	20 and 50 µL
Scintillation vials	
Shaking water bath	
TLC developing chamber	
Whatman Silica Gel 60A K6F TLC Plates	
100mL volumetric flasks	

Chemicals:

Distilled, Deionized water (H_2O)	
Methanol	J.T. Baker or equivalent
Ethyl Acetate	J.T. Baker or equivalent
Acetic Acid	J.T. Baker or equivalent
Sodium Acetate Trihydrate	J.T. Baker or equivalent
Hesperidin Substrate	Sigma Cat.-No. H-5254

Reagents:

0.2 M Acetic Acid (HOAc): Add 1.2 mL HOAc to 95mL water in a 100mL volumetric flask. Dilute to volume with water.

0.2 M Sodium Acetate (NaOAc): Dissolve 2.7 g NaOAc-$3H_2O$ (or 1.6 g NaOAc) in water in a 100mL volumetric flask an dilute to volume with water.

Sodium acetate buffer, pH 3.8: To a 100mL volumetric flask add 44.0mL HOAc and 6.0mL NaOAc. Dilute to volume with water. Make slight adjustments with sodium hydroxide solution or acetic acid if needed.

TLC Developing Solution: Into a 100mL mixing cylinder add 80mL ethyl acetate, 15mL methanol, and 5mL acetic acid. Stopper and mix.

Solutions:

Hesperidinase Stock Solution:
Sigma Enzyme (2.1 units/mL): In a 20mL scintillation vial dissolve 0.3 g hesperidinase in 1mL sodium acetate buffer, pH 3.8. Sonicate to dissolve.

ICN Enzyme (2.1 units/mL): In a 20mL scintillation vial dissolve 0.014 g hesperidinase in 2mL sodium acetate buffer, pH 3.8. Sonicate to dissolve.

Note: Enzyme is not very soluble in buffer, therefore mix vigorously before aliquoting.

Hesperidin Substrate Stock Solution (1 µmol/mL): In a 20mL scintillation vial dissolve 0.006g hesperidin in 10mL sodium acetate buffer, pH 3.8. Sonicate to dissolve.

Assay:

1. Into a 20mL scintillation vial transfer 1mL of the hesperidin substrate solution.
2. Cap the vial and place in a shaking water bath heated to 40°C for 10minutes or until equilibrated to temperature.
3. Add a 1mL aliquot of the Hesperidinase enzyme solution to the 1 mL substrate in the vial cap and place in the shaking water bath.
4. Remove the vial from the shaking water bath after at least 1 hour and spot about 50mL aliquot on the TLC plate. Return vial to water bath.
5. Repeat step 4 with a 25mL aliquot of the hesperidin substrate stock solution.
6. Allow spots to dry completely.
7. Develop the plate in a TLC chamber pre-conditioned with the TLC developing solution.
8. After solvent front has been developed, pull the plate out of the developing chamber and allow to dry in a ventilation hood.
9. Place the plate under UV light and measure the distance traveled by both the hesperidin substrate solution and the enzyme reaction solution. A higher R_f (about 0.8)should be found for the enzyme versus the substrate. This indicates that the enzyme reaction has progressed significantly. If significant spot has not developed repeat from Step 4 after another hour.

Attachment 3:

Sigma Quality Control Test Procedure for ß-Glucosidase (EC 3.2.1.21):

Principle:

ß-D-Glucoside + H_2O $\xrightarrow{\text{ß-Glucosidase}}$ D-Glucose + an Alcohol

Conditions: T = 37° C, pH = 5.0, A_{540nm}, Light path = 1 cm

Method: Colorimetric[1]

Reagents:

A. 100 mM Sodium Acetate Buffer, pH 5.0 at 37° C (Prepare 200 mL in deionized water using Sodium Acetat, Trihydrate, Sigma Prod. No. S-8625. Adjust to pH 5.0 at 37° C with 1 M HCl.)

B. 1 % (w/v) Salicin Substrate Solution (Salicin) (Prepare 50 mL in Reagent A using Salicin, Sigma Prod. No. S-0625).

C. ß-Glucosidase Enzyme Solution
Immediately before use, prepare a solution containing 1.2 - 2.4 units/mL of ß-Glucosidase in cold deionized water.)

D. 16 mM Copper Sulfate, 1300 mM Sodium Sulfate, 226 mM Sodium Carbonate, 190 mM Sodium Bicarbonate, and 43 mM Sodium Potassium Tartrate Solution (Copper Soln) (Prepare 1 liter in deionized water using Cupric Sulfate Pentahydrate, Sigma Prod. No. C-7631, Sodium Bicarbonate, Sigma Prod. No. S-8875, Sodium Sulfate, Anhydrous, Sigma Prod. No. S-9627, Sodium Carbonate, Anhydrous, Sigma Prod. No. S-2127, and Sodium Potassium Tartrate Tetrahydrate, Sigma Prod. No. S-2377.[2])

E. 40 mM Molybdic Acid, 19 mM Arsenic Acid, and 756 mM Sulfuric Acid Solution (Ars-Mol Soln) (Prepare 1 liter in deionized water using Molybic Acid, Ammonium Salt Tetrahydrate, Sigma Prod. No. M-0878, Arsenic Acid, Sodium Salt, Sigma Prod. No. A-6756, and Sulfuric Acid, Sigma Prod. No. S-1526.[3])

F. Glucose Standard Solution (Glucose)
(Use Glucose Standard Solution, Sigma Stock No. 635-100.)

Procedure:

Pipette (in milliliters) the following reagents into suitable test tubes:

	Test	Blank
Reagent B (Salicin)	4.00	4.00

Equilibrate to 37° C. Then add:

Deionized Water	------	1.00
Reagent C (Enzyme)	1.00	------

Mix by inversion and incubate at 37° C for exactly 10 mmutes.

Immediately transfer 1 mL of reaction mixture into a suitable container containing 1 mL of Reagent D as indicated below and proceed with Somogyi's method[2] of assaying reducing sugars. Pipette (in millimeters) the following reagents into suitable containers:

	Test	Test Blank	Std.1	Std.2	Std.3	Std.4	Std.5	Std. Blank
Test Solution	1.00	------	------	------	------	------	------	------
Test Blank Solution	------	1.00	------	------	------	------	------	------
Deionized Water	------	------	0.97	0.95	0.93	0.90	0.80	1.00
Reagent F (Glucose)	------	------	0.03	0.05	0.07	0.10	0.20	------
Reagent D (Copper Soln)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Immediately mix by inversion. Place a marble over the top of the tube and transfer the tubes to a boiling water bath. Incubate for 10 minutes. Remove from the boiling water bath and allow to cool to room temperature. Then add:

Reagent E (Ars-Mol Soln)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Shake or vortex until foaming stops and any precipitate present is dissolved. Then add:

	Test	Blank	Std.1	Std.2	Std.3	Std.4	Std.5	Blank
Deionized Water	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00

Mix and transfer to suitable cuvettes. Obtain the A_{540nm} for Test, Blank and Standards, using a suitable spectrophotometer.

Calculations:

Standard Curve:

$D_{A540nm}Std = A_{540nm}$ Std - A_{540nm}Blank

Prepare a standard curve by plotting the D_{A540nm} of the Standard versus the mmoles of glucose liberated.

Sample Determination:

D_{A540n}mSample = A_{540nm}Test- A_{540nm}Test Blank

Determine the mmoles of glucose liberated using the standard curve.

$$\text{Units/mg enzyme} = \frac{(\mu\text{moles of glucose liberated})\ (5)}{(10)\ (1)\ (\text{mg enzyme/RM})}$$

5 = Volume of Reaction Mix
10 = Time of assay (in minutes) as per Unit Definition
1 = Volume of enzyme assay used in Colorimetric Determination

Unit definition:

One unit will liberate 1.0 µmole of glucose from salicin per minute at pH 5.0 at 37^0 C.

Final assay concentrations:

In a 5.0 mL reaction mix, the final concentrations are 80 mM sodium acetate, 0.8 % (w/v) salicin and 1.2 - 2.4 Units ß-glucosidase.

References:

Somogyi M., (1952) J. Biol. Chem. 195, 19-23
Somogyi M., (1945) J. Biol. Chem. 160, 61-68
Nelson N., (1944) J. Biol. Chem. 153, 375-380.

Notes:

1. The method of assaying for the presence of reducing sugars, described here, is that of Somogyi.

2. Sodium sulfate, Sodium Carbonate, and Sodium Potassium Tartrate are dissolved in approximately 500 mL of deionized water. Cupric Sulfate is dissolved in approximately 100 mL of deionized water and slowly added to the above solution to avoid precipitation. Sodium Bicarbonate is dissolved first in deionized water and then added to the above solution . Dilute the solution to 1 liter. If a precipitate forms, it should be removed by filtration prior to use. Store in an amber bottle and avoid exposure to direct sunlight. Store at room temperature.

3. Molybdic Acid is dissolved in approximately 300 mL of deionized water. Add Sulfuric Acid slowly. Caution this is an exothermic reaction! A solution of arsenic acid is dissolved in approximately 300 mL of deionized water and is added to the above solution. The solution is diluted to a total volume of 1 liter and incubated at 37^0 C for 48 - 72 hours. If a precipitate forms, it should be removed by filtration prior to use. Store in an amber bottle and avoid exposure to direct sunlight. The solution expires six months after preparation. Store at room temperature in an exhaust hood.

4. All products and stock numbers, unless otherwise indicated, are Sigma product and stock numbers.

Attachment 4:

Qualitative Assay of β-Glucosidase Activity by TLC

Purpose: To determine activity of β-Glucosidase by TLC using the substrate Salicin and a normal phase solvent system.

Principal: The enzyme β-Glucosidase will liberate 1.0 μmole of reducing sugar, as glucose, from Salicin per minute at pH 5.0 at 37°C.

Test Substances:

	β-Glucosidase Enzyme	Sigma Cat.-No.: G-0395
or	β-Glucosidase Enzyme	Serva Cat.-No: 22830

Equipment Used:

Eppendorf Pipettors	100μL and 1mL sizes
Drummond Micropipet	25μl and 50 μL
Scintillation vials, 20mL	
Shaking water bath	
TLC developing chamber	
Whatman Silica Gel 60A K6F TLC Plates	
100mL volumetric flasks	

Chemicals:

Distilled, Deionized water (H_2O)	
Methanol	J.T. Baker or equivalent
Ethyl Acetate	J.T. Baker or equivalent
Acetic Acid	J.T. Baker or equivalent
Sodium Acetate Trihydrate	J.T. Baker or equivalent
Salicin Substrate (FW=286)	Sigma Cat.-No.: S-0625

Reagents:

0.2 M Acetic Acid (HOAc): Add 1.2 mL HOAc to 95mL water in a 100mL volumetric flask. Dilute to volume with water.

0.2 M Sodium Acetate (NaOAc): Dissolve 2.7 g NaOAc-$3H_2O$ (or 1.6 g NaOAc) in water in a 100mL volumetric flask an dilute to volume with water.

Sodium acetate buffer, pH 5.0: To a 100mL volumetric flask add 14.8mL HOAc and 35.2mL NaOAc. Dilute to volume with water. Check pH with pH meter. Make slight adjustments with sodium hydroxide solution or acetic acid if needed.

TLC Developing Solution: Into a 100mL mixing cylinder add 80mL ethyl acetate, 15mL methanol, and 5mL acetic acid. Stopper and mix.

Solutions:

β-Glucosidase Stock Solution (140 Units/mL): In a 20mL scintillation vial dissolve 15mg β-Glucosidase in 3mL sodium acetate buffer pH 5.0. Sonicate to dissolve.

Salicin Substrate Stock Solution (140μmol/mL): In a 10mL scintillation vial dissolve 80mg Salicin in 2mL sodium acetate buffer pH 5.0. Sonicate for 5-10 minutes.

Assay:

1. Into a 20mL scintillation vial transfer 1mL of the Salicin substrate solution.
2. Cap the vial and place in a shaking water bath heated to 37°C for 10 minutes or until equilibrated to temperature.
3. Add a 1mL aliquot of the β-Glucosidase enzyme solution to the 1 mL substrate in the vial, cap and place in the shaking water bath.
4. Remove the vial from the shaking water bath after at least 1 hour and spot about 25mL aliquot on the TLC plate. Return vial to water bath.
5. Repeat step 4 with a 50mL aliquot of the Salicin substrate stock solution.
6. Allow spots to dry completely.
7. Develop the plate in a TLC chamber pre-conditioned with the TLC developing solution.
8. After solvent front has been developed, pull the plate out of the developing chamber and allow to dry in a ventilation hood.
9. Place the plate under UV light and measure the distance traveled by both the Salicin substrate solution and the reaction solution. A higher R_f (about 0.8) should be found for the enzyme versus the substrate. This indicates that the enzyme reaction has progressed significantly. If significant spot has not developed, repeat from Step 4 after another hour.