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Study Title

Determination of BAS 490 F and its Metabolites in Apples, Grapes and their Process Commodities

Data Requirement

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

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PR 86-5 DATA CONFIDENTIALITY CLAIM

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

COMPANY BASF CORPORATION / AGRICULTURAL PRODUCTS GROUP

COMPANY AGENT Karen R. Blundell **DATE** Nov. 7, 1996

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Title **Signature**

GOOD LABORATORY PRACTICE STATEMENT

No new data was generated for this report. This document is not required to comply with 40 CFR Part 160, FIFRA Good Laboratory Practices.

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1.0 Introduction

BAS 490 F is a new S trobilurin type fungicide used in cereals, apples, citrus, vine 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 apple fruit, apple processed commodities, grape, grape processed commodities, red pepper, cucumber, tomato and onion. The method first written for these analyses was BASF Method 350/3 (Reference 8). Method 350/3 has been slightly re-written here as Method 350/3-US to include US suppliers of materials and to clarify instructions. The two methods are procedurally identical and differ only in recommended suppliers and the explanations used.

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 plant sample material with methanol. The methanol extract contains the BAS 490 F and the glycosides of BF 490-2 and BF 490-9 from the sample. To this extract an aqueous solution of KH_2PO_4 (5 g/L) is added.

The glycosides of the metabolites BF 490-2 and BF 490-9 which are present in the aqueous extract after evaporation of the methanol, are subjected 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 a following step, the BAS 490 F is converted into the metabolite BF 490-1 by hydrolysis with KOH.

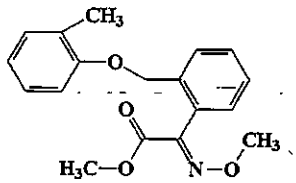
The metabolites are separated from matrix interferences in the extract by phase partitioning into dichloromethane (DCM) followed by a SPE -NH_2-column clean-up step. The chromatography step is based on polarity as well as ion-exchange chromatography. It is followed by a second phase partitioning step 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

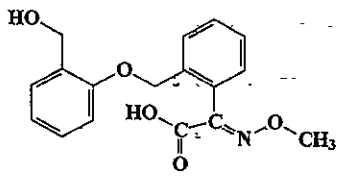
Common name: Kresoxim-methyl (proposed)
Chemical name: Methyl 2-[o-(o-methylphenoxymethyl)phenyl]-2-(methoxyimino) acetate
Structural formula:



Chemical formula: $C_{18}H_{19}NO_4$
Molecular weight: 313.36

Metabolite BF 490-2

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

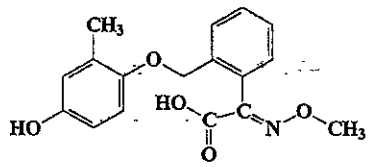


Chemical formula: $C_{17}H_{17}NO_5$
Molecular weight: 315.33

Metabolite BF 490-9

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

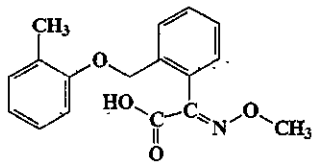
Structural formula:



Chemical formula: $C_{17}H_{17}NO_5$
Molecular weight: 315.33

Metabolite BF 490-1

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

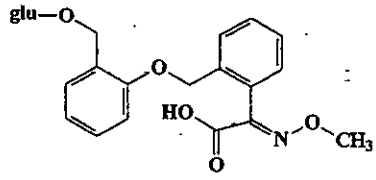


Chemical formula: $C_{17}H_{17}NO_4$
Molecular weight: 299.33

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

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

Structural formula:

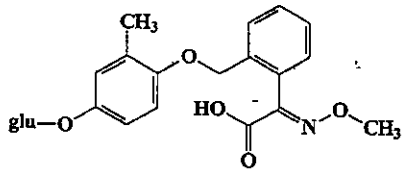


Chemical formula: $C_{23}H_{27}NO_{10}$
Molecular weight: 477.5

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

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 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	
Büchner 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
Graduate Cylinder	100 mL
Erlenmeyer flasks	50 mL
Volumetric flasks	10 mL, 500 mL
Volumetric pipettes	glass, 0.5, 1,2, 4, 5, 10 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
Test tube, tapered tip	10 mL
PTFE syringe filter	0.45µm
Gastight syringe	25 mL
Whatman #4 Filter paper (Qualitative, 90mm circles)	Cat No. 1004-090
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, Phillipsburg, 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
BAKER SPE Amino (NH_2) column packing	J.T. Baker, Cat. No. 7028-00
Plastic SPE valve liners	Supelco, Cat. No. 5-7059

Preparation and Preconditioning of BAKER NH_2-columns:

The glass BAKER columns filled with 0.5 g each of the NH_2-material mentioned previously 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 7.5), 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
KOH 10 mol/L	
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).

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 V 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 VI 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.

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, 12.5 and 125 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 VII 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 VIII Analytical HPLC elution buffer A:
Contains ddH_2O + acetonitrile + formic acid (conc.) 90 + 10 + 0.5 (v/v/v)

Solution IX Analytical HPLC elution buffer B: (IX):
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 (int. diam), Phenomenex, USA Catalog No. 00D-0327-D0

Separation column specification:

C_{18}-Nucleosil (5µ, 100 Å) 250 cm x 3.2 mm (Int. diam.), 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 VII (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 VII.
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 IX (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 IX). Equilibrate the column with Solution VIII.

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	12.5 mg/mL	1.0 mL	0.50 mg/kg
25 g	125.0 mg/mL	1.0 mL	5.00 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 3 minutes using an Ultraturrax or a Polytron at a speed sufficient to homogenized the sample. The homogenate is filtered under vaccum through a Whatman #4 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 gr/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 Analysis of BAS 490 F, BF 490-2 and BF 490-9

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 10 mL by rotary evaporation (under vacuum, 45±5°C) and adjusted to a total mass of 20.0 g with ddH_2O (extract must be absolutely methanol free). The flask should be sonicated with an ultrasonic bath for 10-15 seconds if required to dissolve all residue on the flask.

Note: 20.0 gram adjustment must be made accurately, therefore care should be taken to ensure accuracy.

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. To this flask, 0.5 mL of the ascorbic acid solution (III) and 2.5 mL of the Glucosidase and Hesperidinase stock solution (V) 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 centrifuge the sample 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 The column is washed with 2.5 mL methanol. 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 (VI) 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. 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.

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

The dried sample is redissolved by adding 200 µL acetonitrile, sonicating the sample for a few seconds using an Ultrasonic bath and then mixing on a vortex. 1800 µL acidic ddH_2O (pH 3.4, see 4.2.2) is then added and mixed by using a vortex.

Note: If the sample is cloudy it must be filtered 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 1.0 mL (100 µL acetonitrile and 900 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 (VII)* 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 (VII)* 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 (VIII))* to 90 % acetonitrile *(Analytical HPLC elution buffer B (IX))* 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.

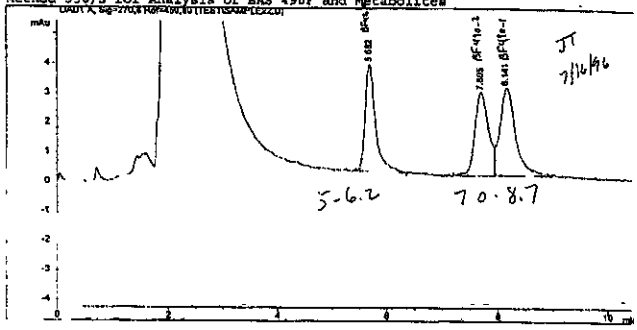
Typical amino column profile for 100 ng injection of BF 490-9, BF 490-2 and BF 490-1. BF 490-1 and BF 490-2 were partially separated.

Data File C:\HPCHEM\1\DATA\TEST\SAMPL822.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\490AM.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	0.2365	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

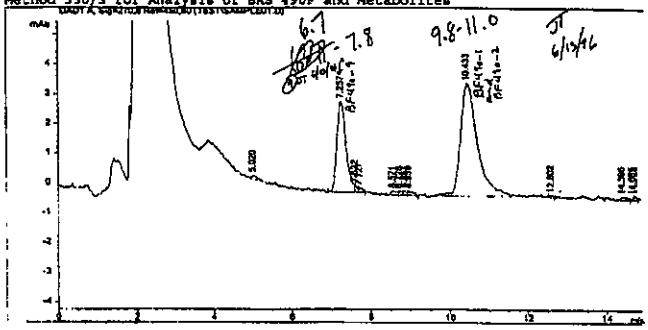
Typical amino column profile for 100 ng injection of BF 490-9, BF 490-2 and BF 490-1. BF 490-1 and BF 490-2 were co-eluted.

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 1:22:24 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!

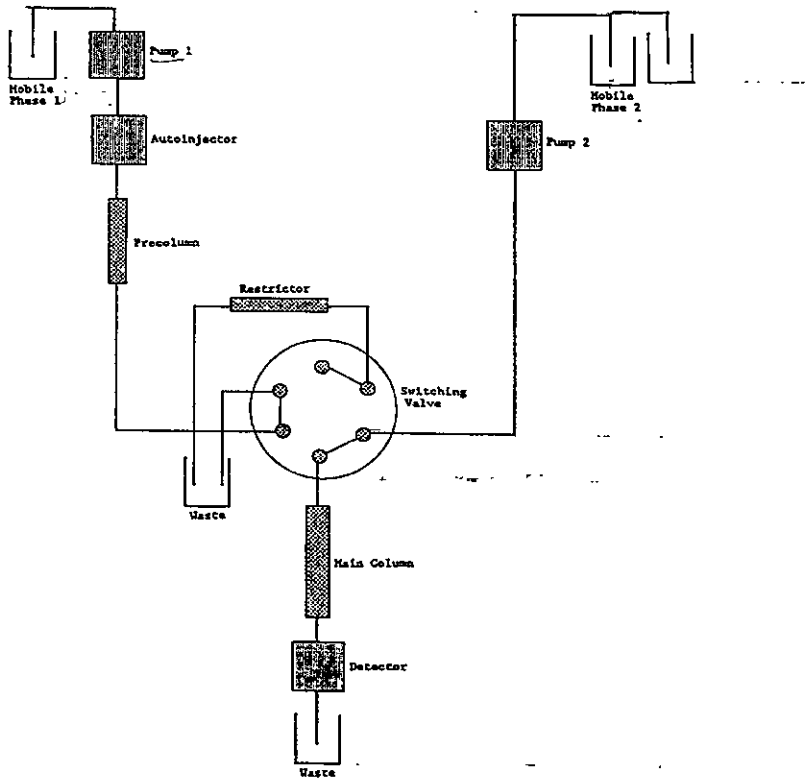
Peak #	RetTime [min]	Type	Width [min]	Area [mAu*s]	Height [mAu]	Area %	
1	5.020	PB	0.0769	9.26227e-1	1.68570e-1	0.5365	
2	7.257	PV	0.2190	49.24270	3.07213	28.5238	BF 490-1
3	7.422	VB	0.0626	9.32713e-1	2.48208e-1	0.5403	
4	7.727	BP	0.0820	1.04071	1.60590e-1	0.6028	
5	8.171	PV	0.1272	1.48572	1.60434e-1	0.8606	
6	8.726	VV	0.0607	7.25124e-1	1.55769e-1	0.4200	
7	8.861	VV	0.0763	8.41145e-1	1.75465e-1	0.4872	
8	8.959	VP	0.1057	1.33170	1.67327e-1	0.7714	
9	10.433	VV	0.3640	113.38899	3.80094	65.6804	BF 490-2 & BF 490-1

Typical gradient profile and control program for analytical HPLC

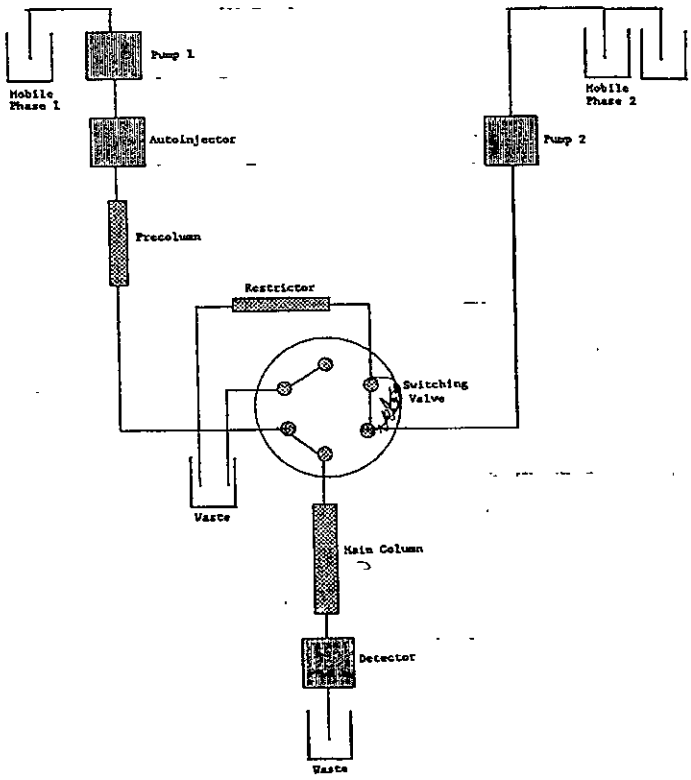
Under these conditions metabolites BF 490-2, BF 490-9 and BF 490-1 will elute at approximately 27.2, 28.1 and 32.6 minutes respectively.

Time [min]	Pre-column Buffer [%]	Flow [mL/min]	Collection window	Buffer A [%]	Buffer B [%]	Flow [mL/min]
0.00	100	0.7		100	0	0.7
6.9	100	0.7	On			
7.3						Stop
8.15	100	0.7	Off			
9.1	100	0.7	On			
9 9				100	0	0.7
10.3	100	0.7	Off			
10.5				100	0	0.7
34.0				30	70	0.7
34.25				0	100	0.7
36.75				0	100	0.7
37.0				100	0	0.7
45.0				100	0	Stop

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).



6.0 Calculation of results

6.1 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 equations I.a - I.c.:

I.a

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

I.b

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

I.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]
$C_{BF\ 490\text{-}1}$	=	Amount of analyte (here: BF 490-1) 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 Metabolite results expressed as BAS 490 F equivalents

The metabolite residue concentrations as calculated in equations I.b and I.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 IV.a and IV.b.

IV.a $$TR_{PE} = \frac{TR_{BF490\text{-}2}}{1.006}$$

IV.b $$TR_{PE} = \frac{TR_{BF490\text{-}9}}{1.006}$$

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

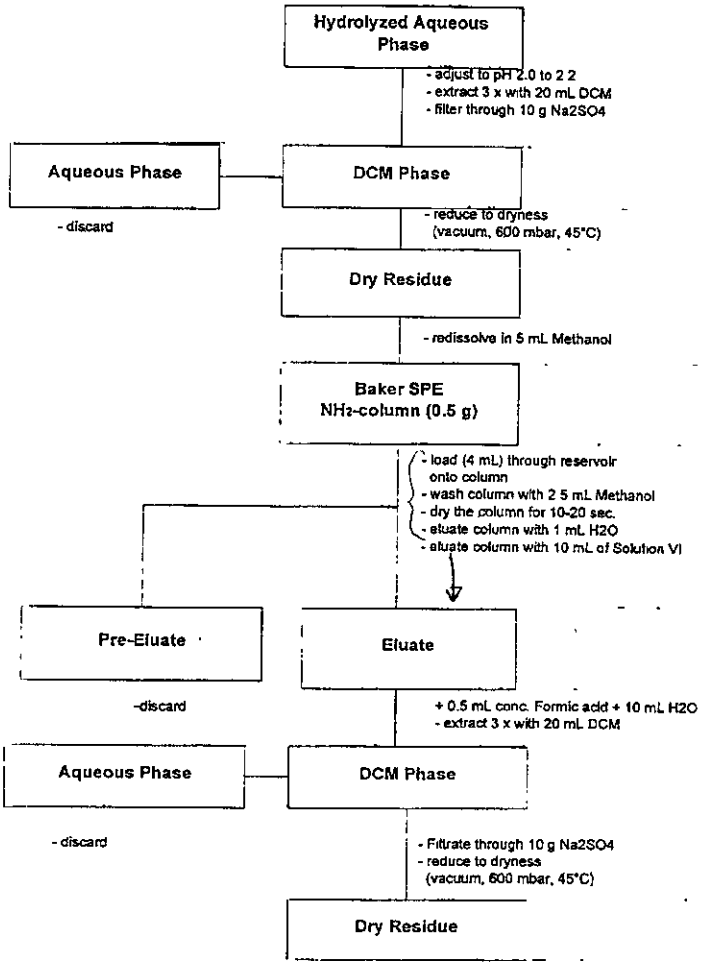
6.3 Calculation of Recovery

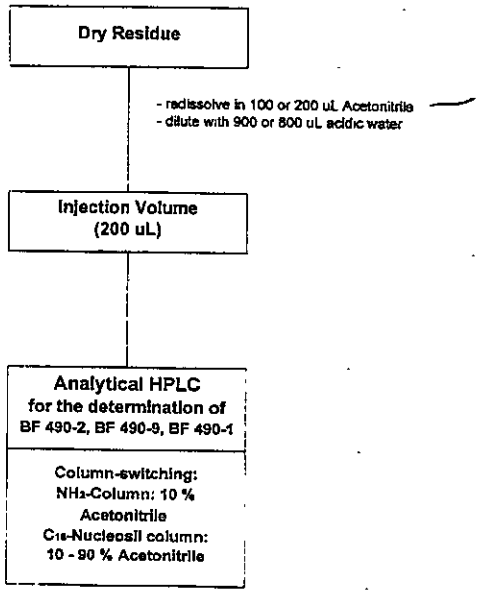
Correct fortification results for residues found in the control sample as follows:

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

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

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

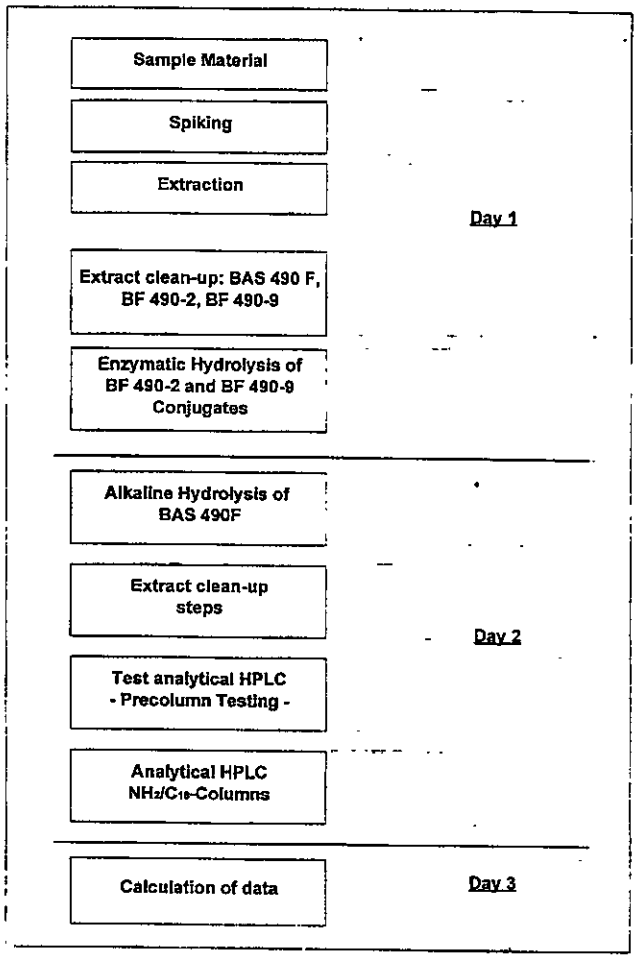

Hydrolyzed Aqueous Phase
- adjust to pH 2.0 to 2.2
- extract 3 x with 20 mL DCM
- filter through 10 g Na2SO4
Aqueous Phase
- discard
DCM Phase
- reduce to dryness (vacuum, 600 mbar, 45°C)
Dry Residue
- redissolve in 5 mL Methanol
Baker SPE NH2-column (0.5 g)
- load (4 mL) through reservoir onto column
- wash column with 2.5 mL Methanol
- dry the column for 10-20 sec.
- eluate column with 1 mL H2O
- eluate column with 10 mL of Solution VI
Pre-Eluate
-discard
Eluate
+ 0.5 mL conc. Formic acid + 10 mL H2O
- extract 3 x with 20 mL DCM
Aqueous Phase
- discard
DCM Phase
- Filtrate through 10 g Na2SO4
- reduce to dryness (vacuum, 600 mbar, 45°C)
Dry Residue


Dry Residue
- redissolve in 100 or 200 uL Acetonitrile
- dilute with 900 or 800 uL acidic water
Injection Volume
(200 uL)
Analytical HPLC
for the determination of
BF 490-2, BF 490-9, BF 490-1
Column-switching:
NH2-Column: 10 %
Acetonitrile
C18-Nucleosil column:
10 - 90 % Acetonitrile

8.0 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 350/3-US is shown in the next page:


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 490F
Extract clean-up steps
Day 2
Test analytical HPLC - Precolumn Testing -
Analytical HPLC NH2/C18-Columns
Calculation of data
Day 3

9.0 Limits of Quantitation

BASF method No. 350/3-US 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 apple, apple processed commodities, grape, and grape processed commodities. With BASF method 350/3-US the limit of quantitation was 0.05 ppm per analyte in these matrices. 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.

10.0 Interferences, 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.

During the development of this technical procedure, various procedures of chemical hydrolyses were tested for the cleavage of the conjugates of BF 490-2 and BF 490-9. In all cases several derivatives and degradational products of each analyte were produced Numbers and yields of these products varied widely, making chemical hydrolysis unsuitable for the use in an analytical method. Enzyme hydrolysis has been found to be the only effective means of releasing the conjugates. The enzymes are stable and have performed very consistently.

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 apples and grapes. No interferences have been found. This study is described in Reference 7.

Reference 7 also contains the results of the radiovalidation study, and a confirmation of the enzyme performance.

11.0 Ruggedness Testing

The method has been used successfully to analyze fortified samples of apples, grapes and their process commodities. The recoveries and standard deviations for BAS 490 F, BF 490-2 and BF 490-9 in apples were 91±17 (n=10), 92±7 (n=10) and 87±9 (n=10), respectively (Reference 8). The recoveries and standard deviations for BAS 490 F, BF 490-2 and BF 490-9 in grapes were 101±7 (n=10), 95±8 (n=10) and 92±8 (n=10), respectively (Reference 8).

12.0 Confirmatory Techniques

For BF 490-2, BF 490-9 and BAS 490 F:

As an alternative to the analytical HPLC with UV-detection a LC/MS-system may be used as confirmatory technique.

For this, the analytical procedure and preparation of the injection volume as described in chapter 5.3.7 should be adopted. However, only a NH_2-column is used for analytical HPLC. The analytical conditions as outlined below allow the quantification of the three metabolites at a level of 0.05 mg/kg each.

HPLC/MS - conditions

Column:	NH_2-column as specified in chapter 4.3.2 (Precolumn specification)
Mode of elution:	Isocratic, with 10 % acetonitrile and 1.0 % formic acid at a pH of about 2.2 (Precolumn elution buffer (VII)
Flow rate:	0.7 mL/min
Injection volume:	50 µL
Split:	95/5
Ionization:	Ion spray
Detection:	Mass 333
Instruments used:	for example: Waters 600-MS (HPLC) and PE-SCIEX API III SN014 (MS)

13 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 Fig. 1, 2 and 3 Methods for the quantitative assays for hesperidnase and ß-Glucosidase are shown in attachments 1 and 3, respectively. Shorter qualitative TLC assays are shown for both enzymes in attachments 2 and 4 respectively.

Figure 1: Biochemical Assay for β-Glucosidase with 2-Nitrophenyl-D-Glycopyranoside

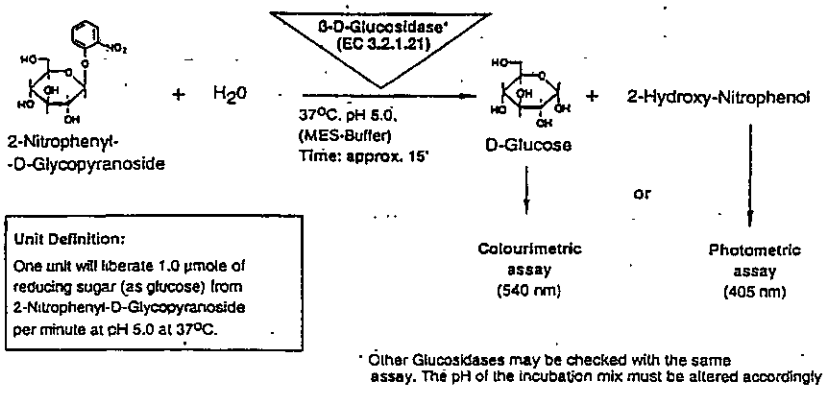


Figure 2: Biochemical Assay for β-Glucosidase with D-(-)-Salicin

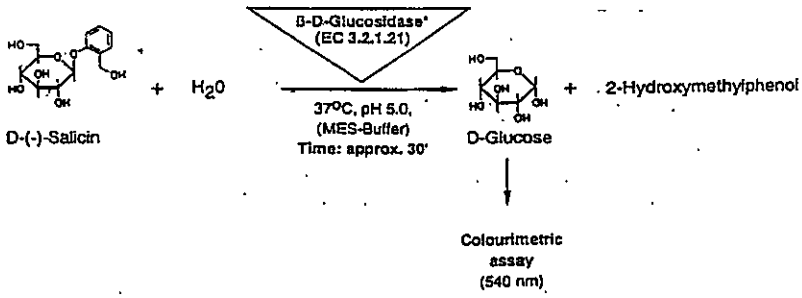
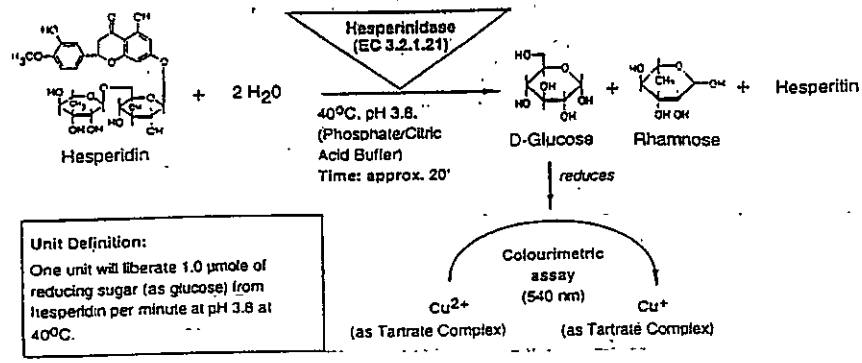


Figure 3: Biochemical assay for Hesperidinase with Hesperidin



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 the following attachments.

14 References

1. Grosshans, FD. The Metabolism of ^{14}C-BAS 490 F (^{14}C-242 009) in Wheat. BASF Registration Document Number 94/10685. August 1994. Limburgerhof, Germany.
2. Nelsen, J., Lewis, C., Wahl, G., Farabee, D. Metabolism of 14C-BAS 490 F in Grapes. BASF Registration Document Number 95/5001. January 1995.
3. Paulick, R. Nature of the Residue of ^{14}C-BAS 490 in Apples. BASF Registration Document Number 96/5175. October 4, 1996.
4. Mackenroth, C. and Krotzky, A. Stability of BAS 490 F in Acetone and BF 490-1 in Acetonitrile. BASF Registration Document Number 94/11098. December 1994. Limburgerhof, Germany.
5. Mackenroth, C. and Krotzky, A. Determination of Stability of BF 490-2, BF 490-6, BF 490-9, and BF 490-18 in Acetonitrile. BASF Registration Document Number 94/11106. December 1994. Limburgerhof, Germany.
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.
7. Movassaghi, S. and Thornton, J. Data in Support of BASF Method 350/3 "Determination of BAS 490 F and It's Metabolites BF 490-2 and BF 490-9 in Crops" BASF Registration Document Number 96/5139. October 7, 1996.
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.

Certification

We, the undersigned, hereby declare that this report provides a true and accurate record of the work conducted.

The author signatures of Method 350/3 can be found in Reference 8.

Author: Sherry Movassaghi — **Sherry Movassaghi** — **Date:** 11/4/96

Approved By: Laura Sears — **Laura Sears**, **Technical Center Leader** — **Date:** 11/4/96

ATTACHMENTS

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 - 0.5 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 37^0 C. Then add:

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

Mix by inversion and incubate at 37^0 C for exactly 10 minutes.

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.

ATTACHMENT 5:

VALIDATION DATA FROM METHOD 350/3 (Reference 8, page 37)

Table 1 Raw data spread sheet: BAS 490 F in grapes

BASF method no. 350/3
Study Code: 31275

Matrix: Grapes
Analyte: BAS 490 F

Queuefile name: HK96029
Lab Journal no. 00825

Sample number	Type	Sample weight (g)	Final dilution(ml)	Sample injected (mg)	Peak height	Std. Conc. (µg/ml)	Substance injected (ng)	Residue (mg/kg)	Spiking level (µg)	Recovery %	Date of extraction	Date of measurem.
9601042	C	25	2	200	0		0.00000	<0.05			16.07.1996	19.07.1996
HK062	S				5.011559	0.025	5.02772					19.07.1996
9601048	F	25	2	200	8.642673		8.60144		1.25	89.5	16.07.1996	19.07.1996
HK064	S				20.52903	0.10	20.29994					19.07.1996
9601047	F	25	2	200	9.325743		9.27372		1.25	98.4	16.07.1996	19.07.1996
9601046	F	25	2	200	8.690357		8.64837		1.25	89.9	16.07.1996	19.07.1996
HK066	S				101.459	0.50	99.95084					19.07.1996
9601049	F	25	2	200	9.716749		9.65854		1.25	100.4	16.07.1996	19.07.1996
9601050	F	25	2	200	10,36644		10 29797		1.25	107.1	16.07.1996	19.07.1996
HK063	S				4.700422	0.025	4.72150					19.07.1996
9601066	F	25	20	20	97.04828		98.91239		125.0	102.9	16.07.1996	20.07.1996
9601067	F	25	20	20	101.5654		103.6747		125.0	107.8	16.07.1996	20.07.1996
HK069	S				240.9875	1.25	249.99710					20.07.1996
9601068	F	25	20	20	100.981		103.04030		125.0	107.2	16.07.1996	20.07.1996
9601069	F	25	20	20	97.39637		99.27776		125 0	103.2	16.07.1996	20.07.1996
HK067	S				100.143	0.50	102.16070					20.07.1996
9601070	F	25	20	20	96.57383		98.41438		125.0	102.4	16.07.1996	20.07.1996
HK070	S				240.2473	1.25	249.22010					20.07.1996
HK065	S				20.55526	0.10	18.62205					20.07.1996

C = Control T = Treated F = Fortified sample S = Standard

Injection volume: 200 µl
Calibration linear
Coeff. of correlation: 1.000/0.9998

Aliquotations 8%
Intercept: 0.9537 e-1/ -2.954
SD+/-: 0.2922 / 0.1894 e+1

Derivatisation factor: 1.04
Slope: 0.9842/ 1.050

Table 2 Raw data spread sheet: BF 490-2 in grapes.

BASF method no. 350/3
Study Code: 31275

Matrix: Grapes
Analyte: BF 490-2

Queuefile name: HK96029
Lab Journal no. 00925

Sample number	Type	Sample weight (g)	Final dilution(ml)	Sample injected (mg)	Peak height	Std. Conc. (µg/ml)	Substance injected (ng)	Residue (mg/kg)	Spiking level (µg)	Recovery %	Date of extraction	Date of measurem.
9601042	C	25	2	200	0		0.00000	<0.05			18.07.1996	19.07.1996
HK062	S				5.089045	0.025	5.016483					19.07.1996
9601046	F	25	2	200	8.386374		8.216446		1.25	82.2	18.07.1996	19.07.1996
HK064	S				20.504	0.10	19.9856					19.07.1996
9601047	F	25	2	200	8 867979		8.686123		1.25	86.9	18.07.1996	19.07.1996
9601048	F	25	2	200	8.44717		8.27749		1.25	82.8	18.07.1996	19.07.1996
HK068	S				102.9038	0.50	100.0023					19.07.1996
9601049	F	25	2	200	9.226799		9.034565		1.25	90.3	18.07.1996	19.07.1998
9601050	F	25	2	200	10.00047		9.78586		1.25	97.9	18.07.1996	19.07.1996
HK063	S				5.067587	0.025	4.995646					19.07.1996
9601066	F	25	20	20	100.9834		101.68390		125.0	101.7	18.07.1996	20.07.1996
9601067	F	25	20	20	100.9452		101.6441		125.0	101.6	18.07.1998	20.07.1996
HK069	S				243.0877	1.25	249.97180					20.07.1996
9601068	F	25	20	20	101.6378		102.36690		125.0	102.4	18.07.1996	20.07.1996
9601069	F	25	20	20	98.99497		99.60865		125.0	99.6	18.07.1996	20.07.1996
HK067	S				101.6712	0.50	102.40180					20.07.1996
9601070	F	25	20	20	99.93067		100.58530		125.0	100.6	18.07.1996	20.07.1998
HK070	S				242.2845	1.25	249.1544				18.07.1996	20.07.1996
HK065	S				21.25263	0.10	18.47198					20.07.1998

C = Control T = Treated F = Fortified sample S = Standard

Injection volume: 200 µl
Calibration: linear
Coeff. of correlation: 1.000/0.9998

Aliquotation: 8%
Intercept: 0.7462 e+1/ -3.709
SD+/-: 0.1586 e-1 / 0.2100 e+1

Derivatisation factor: 1.0
Slope: 0.9711/ 1.044

Table 3 Raw data spread sheet: BF 490-9 in grapes.

BASF method no. 350/3
Study Code: 31275

Matrix: Grapes
Analyte: BF 490-9

Queuefile name: HK96029
Lab Journal no. 00925

Sample number	Type	Sample weight (g)	Final dilution(ml)	Sample injected (mg)	Peak height	Std. Conc. (µg/ml)	Substance injected (ng)	Residue (mg/kg)	Spiking level (µg)	Recovery %	Date of extraction	Date of measurem.
9601042	C	25	2	200	0		0.00000	<0.05			18.07.1996	19.07.1996
HK062	S				5.125999	0.025	4.927984					19.07.1996
9601046	F	25	2	200	8.217096		8.003072		1.25	80.0	18.07.1996	19.07.1996
HK064	S				20.56599	0.10	20.2880					19.07.1996
9601047	F	25	2	200	9.020587		8.802381		1.25	88.0	18.07.1996	19.07.1996
9601048	F	25	2	200	8.084774		7.87144		1.25	78.7	18.07.1996	19.07.1996
HK066	S				100.646	0.50	99.9533					19.07.1996
9601049	F	25	2	200	9.081364		8.862863		1.25	88.6	18.07.1996	19.07.1996
9601050	F	25	2	200	9.785891		9.56374		1.25	95.6	18.07.1996	19.07.1996
HK063	S				5.028248	0.025	4.830739					19.07.1996
9601068	F	25	20	20	98.05289		97.75456		125.0	97.8	18.07.1996	20.07.1996
9601067	F	25	20	20	97.03517		98.7898		125.0	98.8	18.07.1996	20.07.1996
HK069	S				240.2532	1.25	249.69840					20.07.1996
9601066	F	25	20	20	98.71569		98.45295		125.0	98.5	18.07.1996	20.07.1996
9601069	F	25	20	20	92.8092		94.33573		125.0	94.3	18.07.1996	20.07.1996
HK067	S				100.0535	0.50	101.97					20.07.1996
9601070	F	25	20	20	94.10024		95.59708		125.0	95.7	18.07.1996	20.07.1996
HK070	S				240.1555	1.25	249.5934					20.07.1996
HK065	S				21.06428	0.10	18.74011					20.07.1996

C = Control T = Treated F = Fortified sample S = Standard

Injection volume: 200 µl
Calibration: linear
Coeff. of correlation: 1.000/0.9999

Aliquotation: 8%
Intercept: -0.1715/+3.455
SDxy: 0.2439/0.1692 e+1

Derivatisation factor: 1.0
Slope: 0.9948/1.054

Table 4 Raw data spread sheet: BAS 490 F in must (grape juice).

BASF method no. 3503
Study Code: 31275

Matrix: Must
Analyte: BAS 490 F

Queuefile name: WD96028
Lab Journal no. 00954

Sample number	Type	Sample weight (g)	Final dilution (ml)	Sample injected (mg)	Peak height	Std. Conc. (µg/ml)	Substance injected (pg)	Residue (mg/kg)	Spiking level (µg)	Recovery - %	Date of extraction	Date of measurem.
9601043	C	25	2	200	0		0	<0.05			22.05.1996	23.05.1996
WD189	S				5.245209	0.025	5.163950					23.05.1996
9601051	F	25	2	200	10.0553		9.72037		1.25	101.1	22.05.1996	23.05.1996
WD187	S				21.38734	0.10	20.45690					23.05.1996
9601052	F	25	2	200	10.20789		9.86439		1.25	102.6	22.05.1996	23.05.1996
WD185	S				104.4512	0.50	99.15565					23.05.1996
9601054	F	25	2	200	9.624958		9.31264		1.25	96.9	22.05.1996	23.05.1996
9601055	F	25	2	200	9.9151		9.57896		1.25	99.6	22.05.1996	24.05.1996
WD190	S				4.225728	0.025	4.86038					24.05.1996
9601071	F	25	20	20	94.78927		90.00148		1.25	93.6	22.05.1996	24.05.1996
9601072	F	25	20	20	89.74122		94.69221		125.0	98.5	22.05.1996	24.05.1996
9601073	F	25	20	20	89.93196		94.9729		125.0	98.7	22.05.1996	24.05.1996
9601074	F	25	20	20	95.5474		90.71981		125	94.3	22.05.1996	24.05.1996
WD186	S				106.2143	0.50	100.82610					24.05.1996
9601075	F	25	20	20	98.89722		93.89356		125.0	97.6	22.05.1996	24.05.1996
WD188	S				20.4134	0.10	19.53414					24.05.1996
WD191	S				5.077124	0.025	5.00380					24.05.1996
9601053	F	25	2	200	10.14709		9.80734		1.25	102.0	22.05.1996	23.05.1996

Injection volume: 200 µl
Calibration: linear
Coeff. of correlation: 0.9998

Alkylation: 3%
Intercept: 0.1935
SD +/-: 0.6513

Dehydration factor: 1.04
Slope: 0.9474

Table 5 Raw data spread sheet: BF 490-2 in must (grape juice).

BASF method no. 350/3
Study Code: 312275

Matrix: Must
Analyte: BF 490-2

Queuefile name: WD98026
Lab Journal no. 00954

Sample number	Type	Sample weight (g)	Final dilution (ml)	Sample injected (mg)	Peak height	Std. Conc. (µg/ml)	Substance injected (ng)	Residue (mg/kg)	Spiking level (µg)	Recovery %	Date of extraction	Date of measurem.
9601043	C	25	2	250	0		0	<0.05			22.05.1996	23.05.1996
WD189	S				4.918375	0.025	5.129305		1.25	84.5	22.05.1996	23.05.1996
9601051	F	25	2	200	8.338374		8.45381					23.05.1996
WD187	S				20.18571	0.10	19.76557		1.25	83.9	22.05.1996	23.05.1996
9601052	F	25	2	200	8.314848		8.38524					23.05.1996
WD185	S				102.5617	0.50	98.73784		1.25	103.8	22.05.1996	23.05.1996
9601054	F	25	2	200	8.313656		8.38409		1.25	78.8	22.05.1996	23.05.1996
9601055	F	25	2	200	7.784367		7.87668					24.05.1996
WD190	S				4.972219	0.025	5.18073		1.25	86.4	22.05.1996	24.05.1996
9601071	F	25	20	20	89.7191		86.42476		125.0	90.7	22.05.1996	24.05.1996
9601072	F	25	20	20	94.14573		90.67038		125.0	92.7	22.05.1996	24.05.1996
9601073	F	25	20	20	96.23985		92.7343		125	85.2	22.05.1996	24.05.1996
9601074	F	25	20	20	88.48071		85.23849					24.05.1996
WD188	S				105.2535	0.50	101.31950		125.0	88.7	22.05.1996	24.05.1996
9601075	F	25	20	20	82.09156		88.70014					24.05.1996
WD186	S				20.05458	0.10	19.63986					24.05.1996
WD191	S				5.022267	0.025	5.22873					24.05.1996
9601053	F	25	2	200	8.75473		8.80894		1.25	88.1	22.05.1996	23.05.1996

Injection volume: 2000 µl
Calibration: linear
Coeff. of correlation: 0.9997

Algorithm: 3%
Intercept: 0.4140
SDy/x: 0.9506

Derivatisation factor: 1.0
Slope: 0.9587

Table 6 Raw data spread sheet: BF 490-9 in must (grape juice).

BASF method no. 350/3
Study Code: 31275

Matrix: Must
Analyte: BF 490-9

Queuefile name: WD96028
Lab Journal no. 00954

Sample number	Type	Sample weight (g)	Final dilution(ml)	Sample injected (ng)	Peak height	Std. Conc. (µg/ml)	Substance injected (ng)	Residue (mg/kg)	Spiking level (µg)	Recovery %	Date of extraction	Date of measurem.
9601043	C	25	2	200	0		0	<0.05			22.05.1996	23.05.1996
WD189	S				5.639791	0.025	5.153755					23.05.1996
9601051	F	25	2	200	9.866853		9.17222:		1.25	91.7	22.05.1996	23.05.1996
WD187	S				21.20852	0.10	19.95386					23.05.1996
9601052	F	25	2	200	9.224415		8.56141		1.25	85.6	22.05.1996	23.05.1996
WD185	S				105.8337	0.50	100.211					23.05.1996
9601054	F	25	2	200	8.939505		8.29056		1.25	82.9	22.05.1996	23.05.1996
9601055	F	25	2	200	8.815527		8.17271		1.25	81.7	22.05.1996	23.05.1996
WD190	S				5.497932	0.025	5.0189					24.05.1996
9601071	F	25	20	20	89.99944		85.34857		1.25	85.3	22.05.1996	24.05.1996
9601072	F	25	20	20	95.66665		90.73599		125.0	90.7	22.05.1996	24.05.1996
9601073	F	25	20	20	96.99153		91.9860		125.0	92.0	22.05.1996	24.05.1996
9601074	F	25	20	20	90.70635		86.02058		125	86.0	22.05.1996	24.05.1996
WD188	S				105.2284	0.50	99.8257					24.05.1996
9601075	F	25	20	20	97.67532		92.64550		125.0	92.6	22.05.1996	24.05.1996
WD193	S				21.05117	0.10	19.80427					24.05.1996
WD191	S				5.512238	0.025	5.03250					24.05.1996
9601053	F	25	2	200	9.533167		8.85492		1.25	88.5	22.05.1996	23.05.1996

Injection volume: 200µl
Calibration: linear
Coeff. of correlation: 1.000

Calculation: by
Intercept: 0.2076
SD y/x: 0.1676

Dehydration factor: 1:0
Slope: 0.9506

Table 7 Raw data spread sheet: BAS 490 F in apple

BASF method no. 350/3
Study Code: 31275

Matrix: Apple
Analyte: BAS 490 F

Queuefile name: HK96028
Lab Journal no. 00925

Sample number	Type	Sample weight (g)	Final dilution(ml)	Sample injected (mg)	Peak height	Std. Conc. (µg/ml)	Substance injected (ng)	Residue (mg/kg)	Spiking level (µg)	Recovery %	Date of extraction	Date of measurem.
9600954	C	25	2	200	0		0	<0.05			17.07.1996	18.07.1996
HK141	S				5.064011	0.025	3 882507					18.07.1996
HK140	S				238.2158	1.25	249.14330					18.07.1996
9600957	F	25	2	200	8.327961		7,34578		1.25	76.4	17.07.1996	18.07.1996
HK142	S				20.07008	0.10	19,80462					19.07.1996
9600958	F	25	2	200	11.76119		10.98857		1.25	114.3	17.07.1996	19.07.1996
9600959	F	25	2	200	8.238554		7,25092		1.25	75.4	17.07.1996	19.07.1996
HK143	S				97.69678	0.50	102.16950					19.07.1996
9600960	F	25	2	200	10.44273		9.58964		1.25	99.7	17.07.1996	19.07.1996
9600961	F	25	2	200	6.759167		5.68123		1.25	59.1	17.07.1996	19.07.1996
9600972	F	25	20	20	98.47879		95.77705		125.0	99.6	10.07.1996	11.07.1996
9600973	F	25	20	20	92.72933		90.1048		125.0	93.7	10.07.1996	11.07.1996
HK145	S				253.8133	1.25	249.03070					11.07.1996
9600974	F	25	20	20	93.96195		91.32072		125.0	95.0	10.07.1996	11.07.1996
9600975	F	25	20	20	109.005		108.16220		125.0	110.4	10.07.1996	11.07.1996
HK146	S				105.2058	0.50	102.41390					11.07.1996
9600976	F	25	20	20	80.86801		78.40217		125.0	81.5	10.07.1996	11.07.1996
HK147	S				21.83291	0.10	19.96056					11.07.1996
HK144	S				5.044937	0.025	3.59480					10.07.1996

Injection volume: 200 µl
Calibration: linear
Coeff. of correlation: 0.9998/0.9998

Aliquotation: 8%
Intercept: -1.491/-1.283
SD+/-: 1.034/2.091

Derivatisation factor: 1.04
Slope: 1.061/0.9866

Table 8 Raw data spread sheet: BF 490-2 in apple.

BASF method no. 350/3
Study Code: 312276

Matrix: Apple
Analyte: BF 490-2

Queuefile name: HK96028
Lab Journal no. 00925

Sample number	Type	Sample weight (g)	Final dilution(ml)	Sample injected (mg)	Peak height	Std. Conc. (µg/ml)	Substance injected (ng)	Residue (mg/kg) <0.05	Spiking level (µg)	Recovery %	Date of extraction	Date of measurem.
9600954	C	25	2	200	0		0				17.07.1996	18.07.1996
HK141	S				5.20587	0.025	3.745708					18.07.1996
HK140	S				242.0223	1.25	248.99810					18.07.1996
9600957	F	25	2	200	10.3724		9.09531		1.25	91.0	17.07.1996	18.07.1996
HK142	S				20.63632	0.10	19.72589					19.07.1996
9600958	F	25	2	200	10.24604		8.955441		1.25	89.7	17.07.1996	19.07.1996
9600959	F	25	2	200	10.22697		8.945688		1.25	89.5	17.07.1996	19.07.1996
HK143	S				100.5912	0.50	102.6293					19.07.1996
9600960	F	25	2	200	9.97901		8.688699		1.25	86.9	17.07.1996	19.07.1996
9600961	F	25	2	200	10.12067		8.835912		1.25	88.4	17.07.1996	19.07.1996
9600972	F	25	20	20	97.01937		94.25241		125.0	94.3	10.07.1996	11.07.1996
9600973	F	25	20	20	96.91119		94.14565		125.0	94.1	10.07.1996	11.07.1996
HK145	S				254.1089	1.25	248.84870					11.07.1996
9600974	F	25	20	20	94.53177		91.80400		125.0	91.8	10.07.1996	11.07.1996
9600975	F	25	20	20	112.0079		109.00270		125.0	109.0	10.07.1996	11.07.1996
HK146	S				105.803	0.50	102.69840					11.07.1996
9600976	F	25	20	20	87.04543		84.43848		125.0	84.4	10.07.1996	11.07.1996
HK147	S				21.23713	0.10	19.67252					11.07.1996
HK144	S				4.897581	0.025	3.562243					10.07.1996

Injection volume: 200 µl
Calibration: linear
Coeff. of correlation: 0.9998 / 0.9997

Aliquotation: 5%
Intercept: 11.648 / 1.226
SD+/-: 2.1227 / 0.432

Derivatisation factor: 1.0
Slope: 1.058 / 0.9941

Table 9 Raw data spread sheet: BF 490-9 in apple.

BASF method no. 350/3
Study Code: 31275

Matrix: Apple
Analyte: BF 490-9

Queuefile name: HK96028
Lab Journal no. 00925

Sample number	Type	Sample weight (g)	Final dilution(ml)	Sample injected (mg)	Peak height	Std. Conc. (µg/ml)	Substance injected (ng)	Residue (mg/kg)	Spiking level (µg)	Recovery %	Date of extraction	Date of measurem.
9600954	C	25	2	200	0		0	<0.05			17.07.1996	18.07.1996
HK141	S				5.025864	0.025	3.740777					18.07.1996
HK140	S				237.4649	1.25	249.2527					18.07.1998
9600957	F	25	2	200	8.288356		7.16583		1.25	71.7	17.07.1998	18.07.1998
HK142	S				20.57433	0.10	20.16372					19.07.1996
9600958	F	25	2	200	10.31399		9.326316		1.25	93.3	17.07.1996	19.07.1996
9600959	F	25	2	200	9.191036		8.140208		1.25	81.4	17.07.1996	19.07.1896
HK143	S				97.90421	0.50	101.8428					19.07.1996
9600960	F	25	2	200	9.663377		8.850362		1.25	88.5	17.07.1996	19.07.1996
9600961	F	25	2	200	8.802414		7.729728		1.25	77.3	17.07.1996	19.07.1998
9600972	F	25	20	20	90.43694		90.06762		125.0	90.1	10.07.1996	11.07.1996
9600973	F	25	20	20	69.3414		88.95507		125.0	89.0	10.07.1996	11.07.1996
HK145	S				246.6023	1.25	248.65900					11.07.1996
9600974	F	25	20	20	86.9945		88.60278		125.0	88.6	10.07.1996	11.07.1998
9600975	F	25	20	20	103.0362		102.86260		125.0	102.9	10.07.1996	11.07.1996
HK146	S				103.5166	0.50	103.35050					11.07.1998
9600978	F	25	20	20	83.69831		83.42741		125.0	83.4	10.07.1998	11.07.1996
HK147	S				21.07382	0.10	19.62694					11.07.1998
HK144	S				5.059242	0.025	3.38358					10.07.1998

Injection volume: 200 µl
Calibration: linear
Coeff. of correlation: 0.9930/0.9996

Aliquotation: 8%
Intercept: -1.568/-1.774
SD+/-: 1.668/2.814

Derivatisation factor: 1.0
Slope: 1.056/1.019

Table 10 Raw data spread sheet: BAS 490 F in apple juice.

BASF method no. 350/3
Study Code: 31276

Matrix: Apple juice
Analyte: BAS 490 F

Queuefile name: HK96030
Lab Journal no. 00925

Sample number	Type	Sample weight (g)	Final dilution(ml)	Sample injected (mg)	Peak height	Std. Conc. (µg/ml)	Substance injected (ng)	Residue (mg/kg)	Spiking level (µg)	Recovery %	Date of extraction	Date of measurem.
9600955	C	25	2	200	0.5125999	-	0	<0.05			21.06.1996	22.06.1996
HK127	S				6.198717	0.025	3.377782					17.07.1996
HK126	S				246.0468	1.25	248.53070					17.07.1996
9600952	F	25	2	200	10.70261		8.98005		1.25	93.4	16.07.1996	17.07.1996
HK128	S				20.94507	0.10	19.40558					17.07.1996
9600953	F	25	2	200	9.9051		8.16829		1.25	85.0	16.07.1996	17.07.1996
9600964	F	25	2	200	11.23786		9.52487		1.25	99.1	16.07.1996	17.07.1996
HK129	S				103.7455	0.50	103.68590					17.07.1996
9600965	F	25	2	200	11.7147		10.01023		1.25	104.1	16.07.1996	17.07.1996
9600966	F	25	2	200	9.372234		7.62590		1.25	79.3	16.07.1996	17.07.1996
9600977	F	25	20	20	105.1104		96.87740		125.0	100.7	21.06.1996	22.06.1996
9600978	F	25	20	20	103.6596		95.5450		125.0	99.3	21.06.1996	22.06.1996
HK131	S				272.0332	1.25	250.18190					22.06.1996
9600979	F	25	20	20	100.9843		93.06958		125.0	96.7	21.06.1996	22.06.1996
9600980	F	25	20	20	100.6091		92.74330		125.0	98.4	21.06.1996	22.06.1996
HK132	S				107.9667	0.50	99.50063					22.06.1996
9600981	F	25	20	20	102.21		94.21366		125.0	97.9	21.06.1996	22.06.1996
HK133	S				21.58761	0.10	20.16875					22.06.1996
HK130	S				5.233268	0.025	5.14868					22.06.1996

Injection volume: 200 µl
Calibration: linear
Coeff. of correlation: 0.9995/1.000

Aliquotation: 8%
Intercept: -1.914/0.3423
SD+/-: 3.050/0.4081

Derivatisation factor: 1.04
Slope: 1.018/0.0184

Table 11 Raw data spread sheet: BF 490-2 in apple juice.

BASF method no. 350/3
Study Code: 31275

Matrix: Apple juice
Analyte: BF 490-2

Queuefile name: HK96030
Lab Journal no. 00925

Sample number	Type	Sample weight (g)	Final dilution (ml)	Sample injected (mg)	Peak height	Std. Conc. (µg/ml)	Substance injected (ng)	Residue (mg/kg)	Spiking level (µg)	Recovery %	Date of extraction	Date of measurem.
9600955	C	25	2	200	0		0	<0.05			21.08.1996	22.06.1996
HK127	S				5.304813	0.025	3.874346					17.07.1996
HK126	S				248.5573	1.25	249.02550					17.07.1996
9600962	F	25	2	200	8.827448		7.42448		1.25	74.2	16.07.1996	17.07.1996
HK128	S				20.92481	0.10	19.61626					17.07.1996
9600963	F	25	2	200	8.519688		7.114516		1.25	71.1	16.07.1996	17.07.1996
9600964	F	25	2	200	8.352995		6.946320		1.25	69.5	16.07.1996	17.07.1996
HK129	S				103.1508	0.50	102.4839					17.07.1996
9600965	F	25	2	200	9.313822		7.914647		1.25	79.1	16.07.1996	17.07.1996
9600968	F	25	2	200	9.194613		7.794507		1.25	77.9	16.07.1996	17.07.1996
9600977	F	25	20	20	99.51949		95.91150		125.0	95.9	21.08.1996	22.06.1996
9600978	F	25	20	20	99.46823		95.86231		125.0	95.9	21.08.1996	22.06.1996
HK131	S				260.4711	1.25	250.38410					22.06.1996
9600979	F	25	20	20	98.84119		95.26051		125.0	95.3	21.08.1996	22.06.1996
9600980	F	25	20	20	99.30015		95.70099		125.0	95.7	21.08.1996	22.06.1996
HK132	S				102.6654	0.50	98.93080					22.06.1996
9600981	F	25	20	20	99.17498		95.58086		125.0	95.6	21.08.1996	22.06.1996
HK133	S				20.83659	0.10	20.39594					22.06.1996
HK130	S				5.096197	0.025	5.28917					22.06.1996

Injection volume: 200 µl
Calibration: linear
Coeff. of correlation: 0.9998/ 1.000

Aliquotation: 8%
-Intercept: -1.472/0.3981
SD+/-: 2.056/ 0.8750

Derivatisation factor: 1.0
Slope: 1.008/ 0.9597

Table 12 Raw data spread sheet: BF 490-9 in apple juice.

BASF method no. 350/3
Study Code: 31275

Matrix: Apple juice
Analyte: BF 490-9

Queuefile name: HK96030
Lab Journal no. 00925

Sample number	Type	Sample weight (g)	Final dilution (ml)	Sample injected (mg)	Peak height	Std. Conc. (µg/ml)	Substance injected (ng)	Residue (mg/kg)	Spiking level (µg)	Recovery %	Date of extraction	Date of measurem.
9600955	C	25	2	200	0		0	<0.05			21.06.1996	22.06.1996
HK127	S				5.159378	0.025	3.809791					17.07.1996
HK128	S				243.3324	1.25	248.90740					17.07.1996
9600962	F	25	2	200	8.798838		7.55506		1.25	75.6	16.07.1996	17.07.1996
HK128	S				20.40386	0.10	19.49749					17.07.1996
9600963	F	25	2	200	8.608103		7.358783		1.25	73.8	16.07.1996	17.07.1996
9600964	F	25	2	200	8.960962		7.721901		1.25	77.2	16.07.1996	17.07.1996
HK129	S				101.3386	0.50	102.7853					17.07.1996
9600965	F	25	2	200	10.22697		9.024711		1.25	90.2	16.07.1996	17.07.1996
9600966	F	25	2	200	9.607077		8.385801		1.25	83.9	16.07.1996	17.07.1996
9600977	F	25	20	20	96.25077		95.34904		125.0	95.3	21.06.1996	22.06.1996
9600978	F	25	20	20	96.00997		95.11131		125.0	95.1	21.06.1996	22.06.1996
HK131	S				252.8905	1.25	249.985					22.06.1996
9600979	F	25	20	20	93.57333		92.70585		125.0	92.7	21.06.1996	22.06.1996
9600980	F	25	20	20	95.94202		95.04423		125.0	95.0	21.06.1996	22.06.1996
HK132	S				101.0096	0.50	100.047					22.06.1996
9600981	F	25	20	20	96.23885		95.33727		125.0	95.3	21.06.1996	22.06.1996
HK133	S				19.871	0.10	19.94635					22.06.1996
HK130	S				4.752874	0.025	5.021622					22.06.1996

Injection volume: 200 µl
Calibration: linear
Coeff. of correlation: 0.9978/1.000

Aliquotation: 8%
Intercept: -1.600/0.3295
SD+/-: 2.304/0.9375e-1

Derivatisation factor: 1.0
Slope: 1.029/0.9872

Table 13 Recoveries of BAS 490 F, BF 490-2 and BF 490-9 in grapes.

Queuefile name: HK96029 Recovery of BAS 490 F in grapes

Analyte added (ppm)	analyte added (µg)	Analyte found	Recovery (%)	Mean Recovery (%)	Std Dev. ±	Coef of var.	Repeatability (%)
control							
0.05	1.25	1.12	89.5				
0.05	1.25	1.21	96.4				
0.05	1.25	1.12	89.9				
0.05	1.25	1.26	100.4				
0.05	1.25	1.34	107.1				
				96.7	7.4	7.7	17.6
control							
5.0	125.0	128.6	102.9				
5.0	125.0	134.8	107.8				
5.0	125.0	134.0	107.2				
5.0	125.0	129.0	103.2				
5.0	125.0	128.0	102.4				
				104.7	2.6	2.5	5.4

Queuefile name: HK96029 Recovery of BF 490-2 in grapes

Analyte added (ppm)	analyte added (µg)	Analyte found	Recovery (%)	Mean Recovery (%)	Std Dev. ±	Coef of var.	Repeatability (%)
control							
0.05	1.25	1.03	82.2				
0.05	1.25	1.09	86.9				
0.05	1.25	1.04	82.2				
0.05	1.25	1.13	90.3				
0.05	1.25	1.22	97.9				
				88.0	6.4	7.3	15.7
control							
5.0	125.0	127.1	101.7				
5.0	125.0	127.0	101.6				
5.0	125.0	128.0	102.4				
5.0	125.0	124.5	99.6				
5.0	125.0	125.8	100.6				
				101.2	1.1	1.1	2.8

Queuefile name: HK96029 Recovery of BF 490-9 in grapes

Analyte added (ppm)	analyte added (µg)	Analyte found	Recovery (%)	Mean Recovery (%)	Std Dev. ±	Coef of var.	Repeatability (%)
control							
0.05	1.25	1.00	80.0				
0.05	1.25	1.10	88.0				
0.05	1.25	0.98	78.7				
0.05	1.25	1.11	88.6				
0.05	1.25	1.20	95.6				
				86.2	6.9	8.0	16.9
control							
5.0	125.0	122.3	97.8				
5.0	125.0	123.5	98.8				
5.0	125.0	123.1	98.5				
5.0	125.0	117.9	94.3				
5.0	125.0	119.6	95.7				
				97.0	1.9	2.0	4.5

Table 14 Recoveries of BAS 490 F, BF 490-2 and BF 490-9 in must (grape juice).

Queuefile name: WD96024 Recovery of BAS 490 F in must (grape juice).

Analyte added (ppm)	analyte added (µg)	Analyte found	Recovery (%)	Mean Recovery (%)	Std Dev. ±	Coef of var.	Repeatability (%)
control							
0.05	1.25	1.26	101.1				
0.05	1.25	1.28	102.6				
0.05	1.25	1.21	96.9				
0.05	1.25	1.25	99.6				
0.05	1.25	1.28	102.0				
				100.4	2.3	2.3	5.7
control							
5.0	125.0	117.0	93.6				
5.0	125.0	123.1	98.5				
5.0	125.0	123.4	98.7				
5.0	125.0	117.9	94.3				
5.0	125.0	122.0	97.6				
				96.5	2.4	2.5	5.1

Queuefile name: WD96024 Recovery of BF 490-2 in must (grape juice).

Analyte added (ppm)	analyte added (µg)	Analyte found	Recovery (%)	Mean Recovery (%)	Std Dev. ±	Coef of var.	Repeatability (%)
control							
0.05	1.25	1.06	84.5				
0.05	1.25	1.05	83.9				
0.05	1.25	1.05	83.8				
0.05	1.25	0.99	78.8				
0.05	1.25	1.10	88.1				
				83.8	3.3	4.0	9.3
control							
5.0	125.0	108.0	86.4				
5.0	125.0	113.4	90.7				
5.0	125.0	115.9	92.7				
5.0	125.0	106.5	85.2				
5.0	125.0	110.9	88.7				
				88.7	3.1	3.5	7.5

Queuefile name: WD96024 Recovery of BF 490-9 in must (grape juice).

Analyte added (ppm)	analyte added (µg)	Analyte found	Recovery (%)	Mean Recovery (%)	Std Dev ±	Coef of var.	Repeatability (%)
control							
0.05	1.25	1.15	91.7				
0.05	1.25	1.07	85.6				
0.05	1.25	1.04	82.9				
0.05	1.25	1.02	81.7				
0.05	1.25	1.11	88.5				
				86.1	4.1	4.8	10.0
control							
5.0	125.0	106.6	85.3				
5.0	125.0	113.4	90.7				
5.0	125.0	115.0	92.0				
5.0	125.0	107.7	86.0				
5.0	125.0	115.8	92.6				
				89.3	3.4	3.8	7.3

Table 15 Recoveries of BAS 490 F, BF 490-2 and BF 490-9 in apples.

Queuefile name: HK96028 Recovery of BAS 490 F in apple

Analyte added (ppm)	analyte added (µg)	Analyte found	Recovery (%)	Mean Recovery (%)	Std Dev. ±	Coef of var.	Repeatability (%)
control							
0.05	1.25	0.96	76.4				
0.05	1.25	1.43	114.3				
0.05	1.25	0.94	75.4				
0.05	1.25	1.25	99.7				
0.05	1.25	0.74	59.1				
				85.0	21.9	25.7	55.2
control							
5.0	125.0	124.5	99.6				
5.0	125.0	117.1	93.7				
5.0	125.0	118 8	95.0				
5.0	125.0	138.0	110.4				
5.0	125.0	101.9	81.5				
				96.0	10.5	10.9	28.9

Queuefile name: HK96028 Recovery of BF 490-2 in apple

Analyte added (ppm)	analyte added (µg)	Analyte found	Recovery (%)	Mean Recovery (%)	Std Dev. ±	Coef of var	Repeatability (%)
control							
0.05	1.25	1.14	91.0				
0.05	1.25	1.12	89.7				
0.05	1.25	1.12	89.5				
0.05	1.25	1 09	86 9				
0.05	1.25	1.11	88.4				
				89.1	1.5	1.7	4.1
control							
5.0	125.0	117.9	94.3				
5.0	125.0	117.6	94.1				
5.0	125.0	114.8	91 8				
5.0	125.0	136.3	109.0				
5.0	125.0	105.5	84.4				
				94.7	8.9	9.4	24.6

Queuefile name: HK96028 Recovery of BAS 490-9 in apple

Analyte added (ppm)	analyte added (µg)	Analyte found	Recovery (%)	Mean Recovery (%)	Std Dev. ±	Coef of var.	Repeatability (%)
control							
0.05	1.25	0.90	71.7				
0.05	1.25	1.17	93.3				
0.05	1.25	1.02	81.4				
0.05	1.25	1.11	88.5				
0.05	1.25	0.97	77 3				
				82 4	8.6	10.5	21.6
control							
5.0	125.0	112.6	90.1				
5.0	125.0	111.3	89.0				
5.0	125.0	110.8	88.6				
5.0	125.0	128 6	102.9				
5.0	125.0	104.3	83.4				
				90.8	7.2	8.0	19.6

Table 16 Recoveries of BAS 490 F, BF 490-2 and BF 490-9 in apple juice.

Queuefile name: HK96028 Recovery of BAS 490 F in apple juice

Analyte added (ppm)	analyte added (µg)	Analyte found	Recovery (%)	Mean Recovery (%)	Std Dev. ±	Coef of var.	Repeatability (%)
control							
0.05	1.25	1.17	93.4				
0.05	1.25	1.06	85.0				
0.05	1.25	1.24	99.1				
0.05	1.25	1.30	104.1				
0.05	1.25	0.99	79.3				
				92.2	10.1	11.0	24.8
control							
5.0	125.0	125.9	100.7				
5.0	125.0	124.1	99.3				
5.0	125.0	120.9	96.7				
5.0	125.0	120.5	96.4				
5.0	125.0	122.4	97.9				
				98.2	1.8	1.8	4.3

Queuefile name: HK96028 Recovery of BF 490-2 in apple juice

Analyte added (ppm)	analyte added (µg)	Analyte found	Recovery (%)	Mean Recovery (%)	Std Dev. ±	Coef of var.	Repeatability (%)
control							
0.05	1.25	0.93	74.2				
0.05	1.25	0.89	71.1				
0.05	1.25	0.87	69.5				
0.05	1.25	0.99	79.1				
0.05	1.25	0.97	77.9				
				74.4	4.2	5.6	9.6
control							
5.0	125.0	119.9	95.9				
5.0	125.0	119.9	95.9				
5.0	125.0	119.1	95.3				
5.0	125.0	119.6	95.7				
5.0	125.0	119.5	95.6				
				95.7	0.2	0.3	0.6

Queuefile name: HK96028 Recovery of BAS 490-9 in apple juice

Analyte added (ppm)	analyte added (µg)	Analyte found	Recovery (%)	Mean Recovery (%)	Std Dev. ±	Coef of var.	Repeatability (%)
control							
0.05	1.25	0.95	75.6				
0.05	1.25	0.92	73.6				
0.05	1.25	0.97	77.2				
0.05	1.25	1.13	90.2				
0.05	1.25	1.05	83.9				
				80.1	6.9	8.5	16.6
control							
5.0	125.0	119.1	95.3				
5.0	125.0	118.9	95.1				
5.0	125.0	115.9	92.7				
5.0	125.0	118.8	95.0				
5.0	125.0	119.1	95.3				
				94.7	1.1	1.2	2.6

Table 17 Summary of recoveries for BAS 490 F, BF 490-2 and BF 490-9 in grapes, must (grape juice), apple, and apple juice.

Summary of the recovies for BAS 490 F

Matrix	Replicatess	Minimum recovery (%)	Maximum recovery (%)	Mean recovery (%)	Std. Dev. ±	Coeff.of var.
Grapes	10	89.5	107.8	100.7	6.7	6.7
Must (grape juice)	10	93.6	102.6	98.5	3.0	3.1
Apple	10	59.1	114.3	90.5	17.2	19.0
Apple juice	10	79.3	104.1	95.2	7.5	7.9

Summary of the recoveries for BF 490-2

Matrix	Replicatess	Minimum recovery (%)	Maximum recovery (%)	Mean recovery (%)	Std. Dev. ±	Coeff.of var.
Grapes	10	82.2	102.4	94.6	8.2	8.7
Must (grape juice)	10	78.8	92.7	86.3	4.0	4.6
Apple	10	84.4	94.3	91.9	6.7	7.3
Apple juice	10	69.5	95.3	84.0	11.6	13.6

Summary of the recoveries for BF 490-9

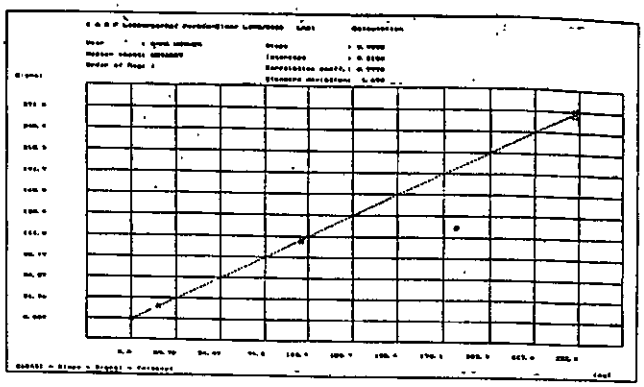
Matrix	Replicatess	Minimum recovery (%)	Maximum recovery (%)	Mean recovery (%)	Std. Dev. ±	Coeff.of var.
Grapes	10	78.7	98.8	91.6	7.5	8.1
Must (grape juice)	10	81.7	92.6	87.7	3.9	4.5
Apple	10	71.7	102.9	86.6	8.7	10.0
Apple juice	10	73.6	95.3	87.4	9.0	10.3

Attachment 6

Chromatograms from Method 350/3 (Reference 8, page 37)

Figure 1 Typical calibration curves for BAS 490 F, BF 490-2 and BF 490-9.

BAS 490 F



BF 490-2

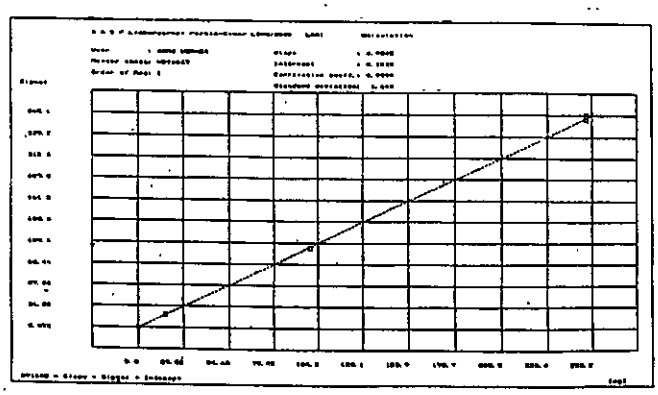


Figure 1 Typical calibration curves for BAS 490 F, BF 490-2 and BF 490-9 (continued).

BF 490-9

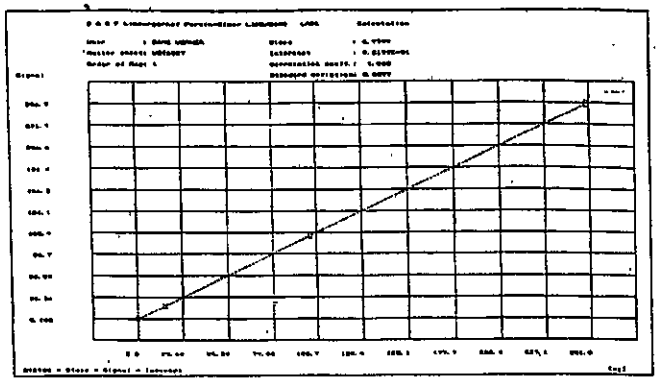


Figure 2 Typical standard chromatogram for BF 490-1, BF 490-2 and BF 490-9 (0.1 mg/kg).

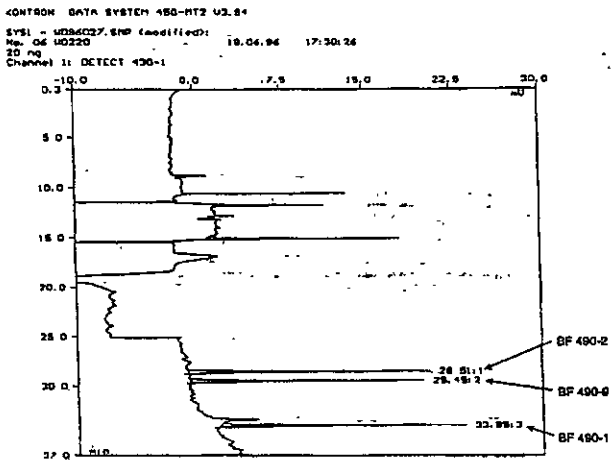


Figure 3 Method 350/3 in grape: Chromatogram of control and 5 fortified sample at 0.05 ppm.

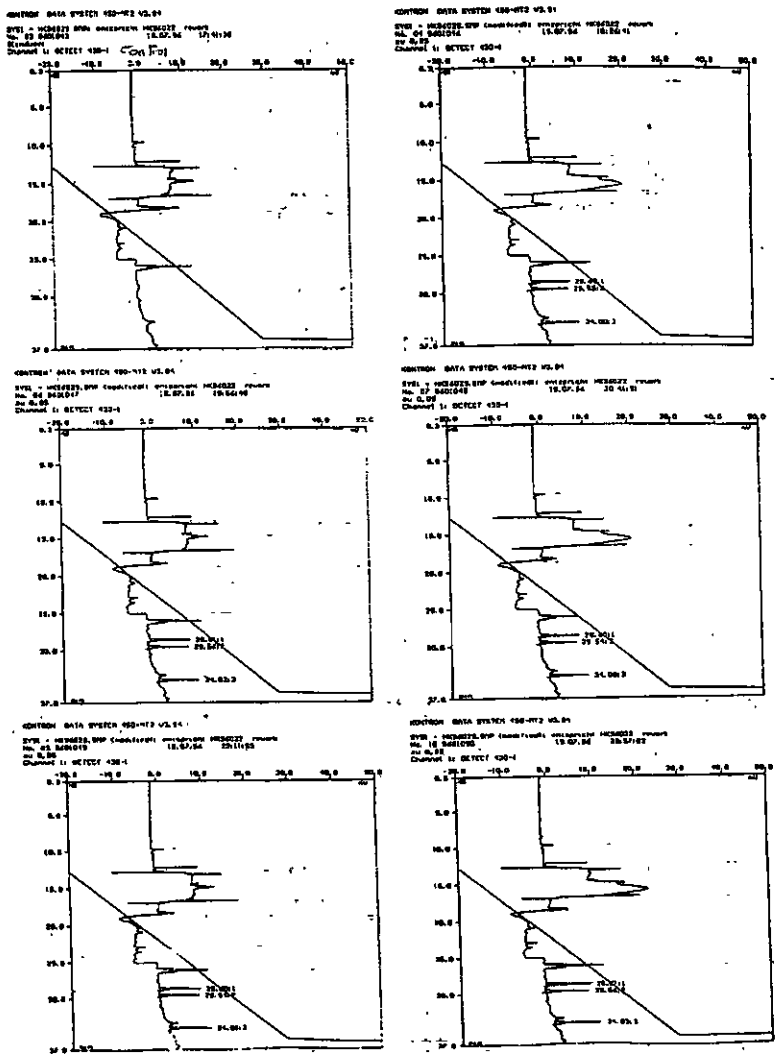


Figure 4 Method 350/3 in grape: Chromatogram of control and 5 fortified sample at 5.0 ppm.

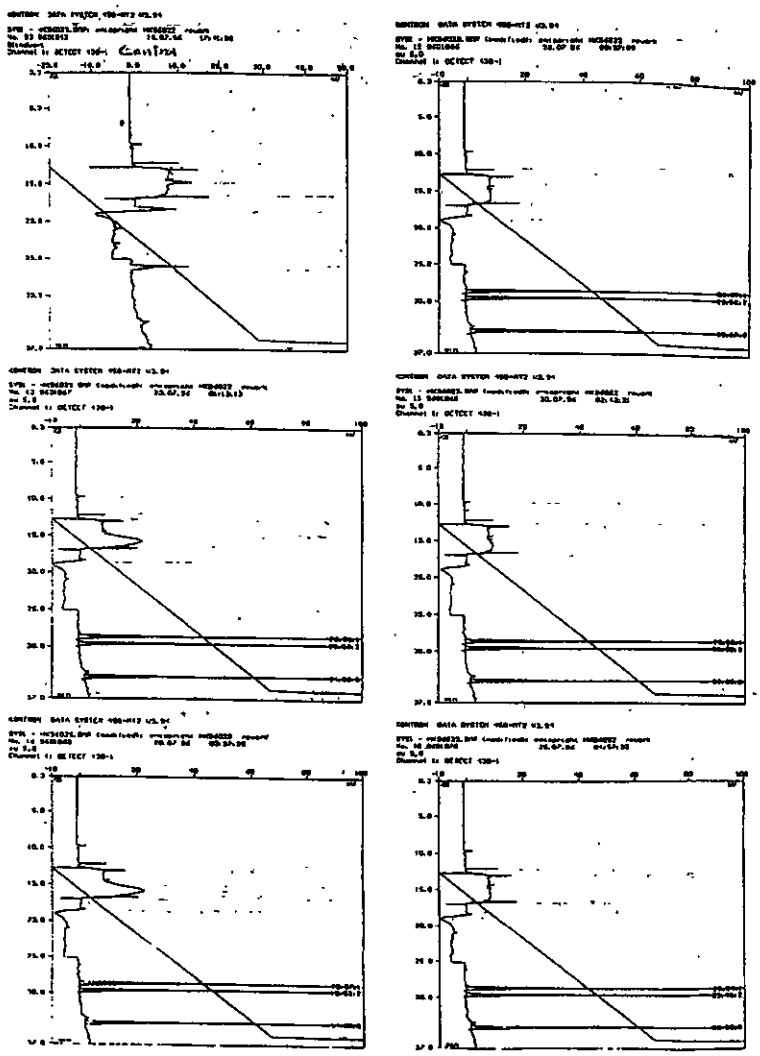


Figure 5 Method 350/3 in must(grape juice): Chromatogram of control and 5 fortified sample at 0.05 ppm.

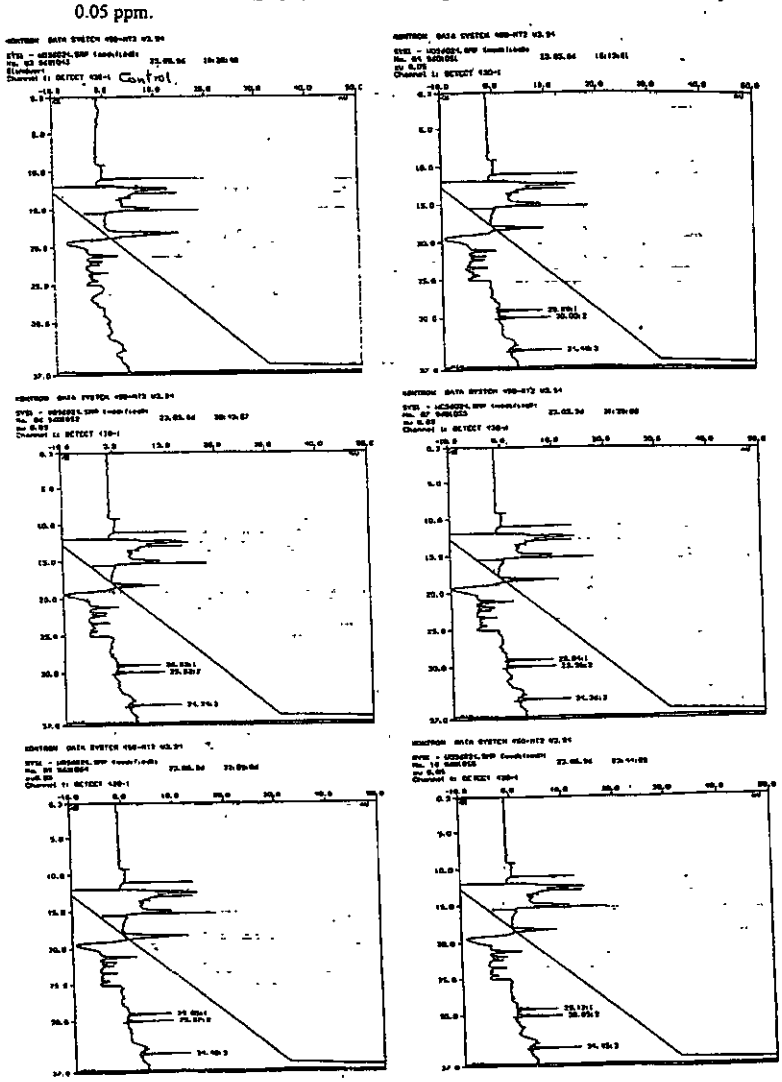


Figure 6 Method 350/3 in must(grape juice): Chromatogram of control and 5 fortified sample at 5.0 ppm.

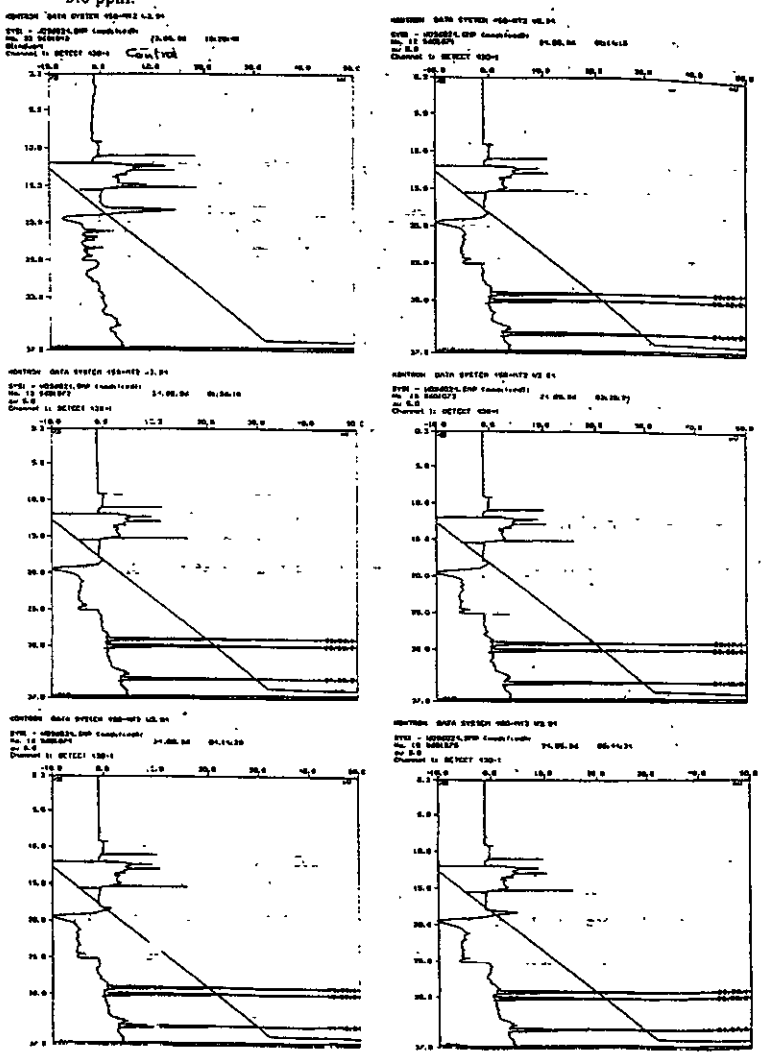


Figure 7 Method 350/3 in apple: Chromatogram of control and 5 fortified sample at 0.05 ppm.

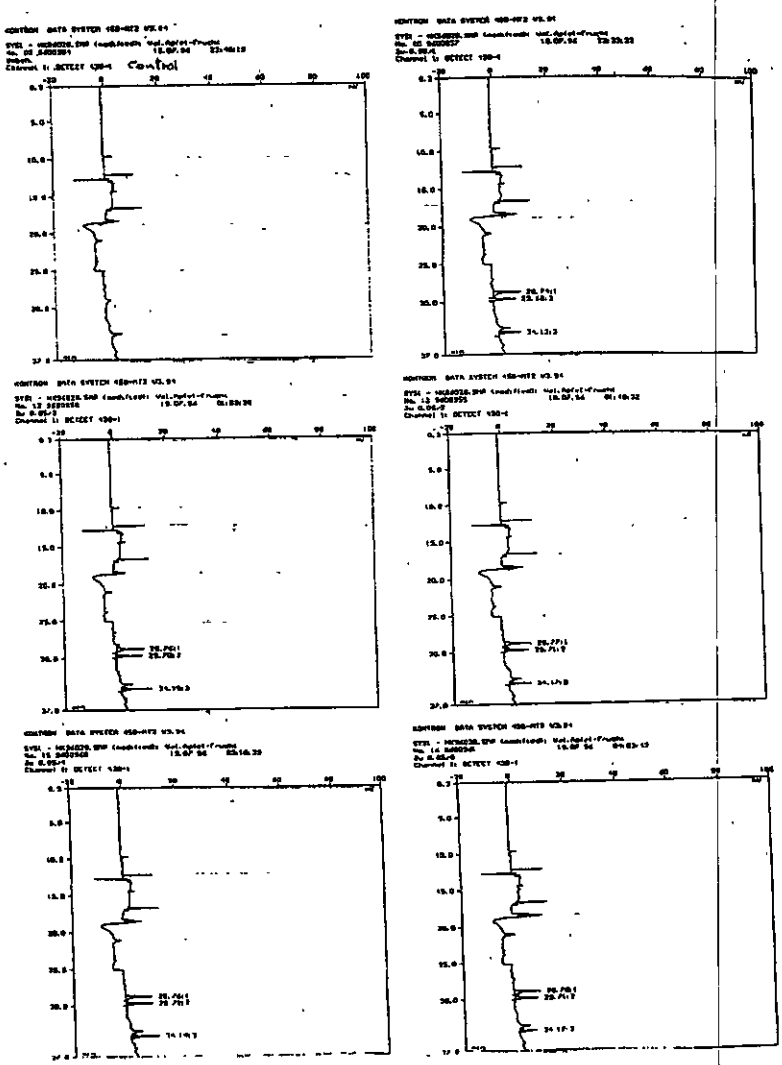


Figure 8 Method 350/3 in apple: Chromatogram of control and 5 fortified sample at 5.0 ppm.

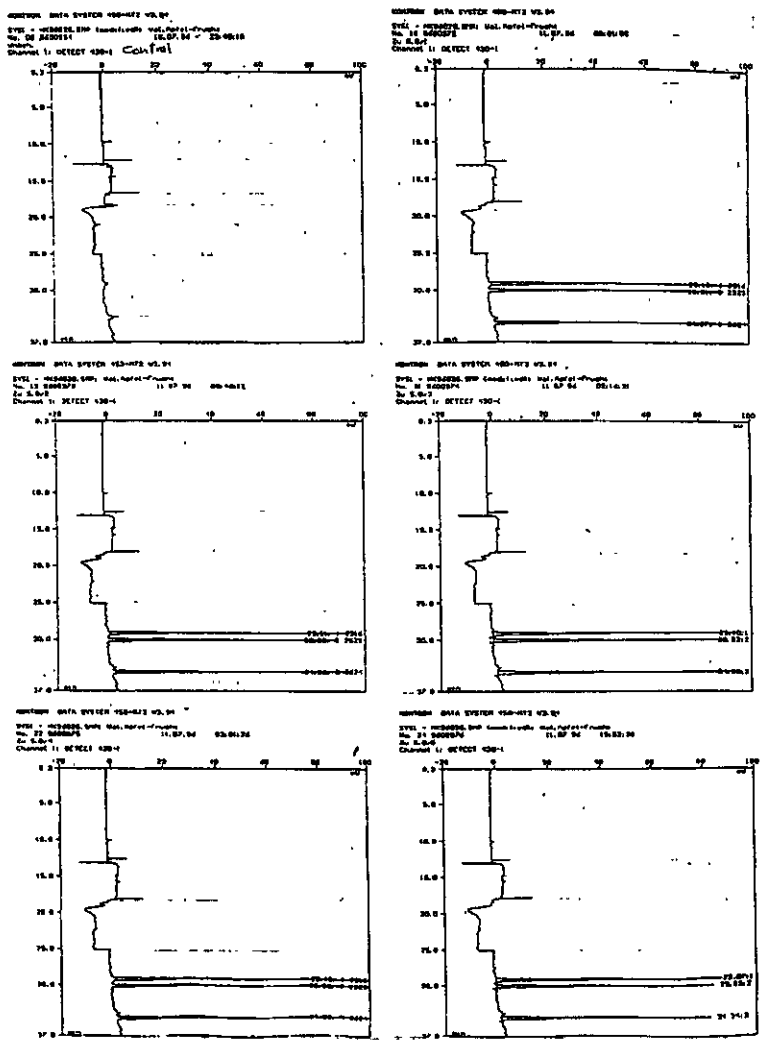


Figure 9 Method 350/3 in apple juice: Chromatogram of control and 5 fortified sample at 0.05 ppm.

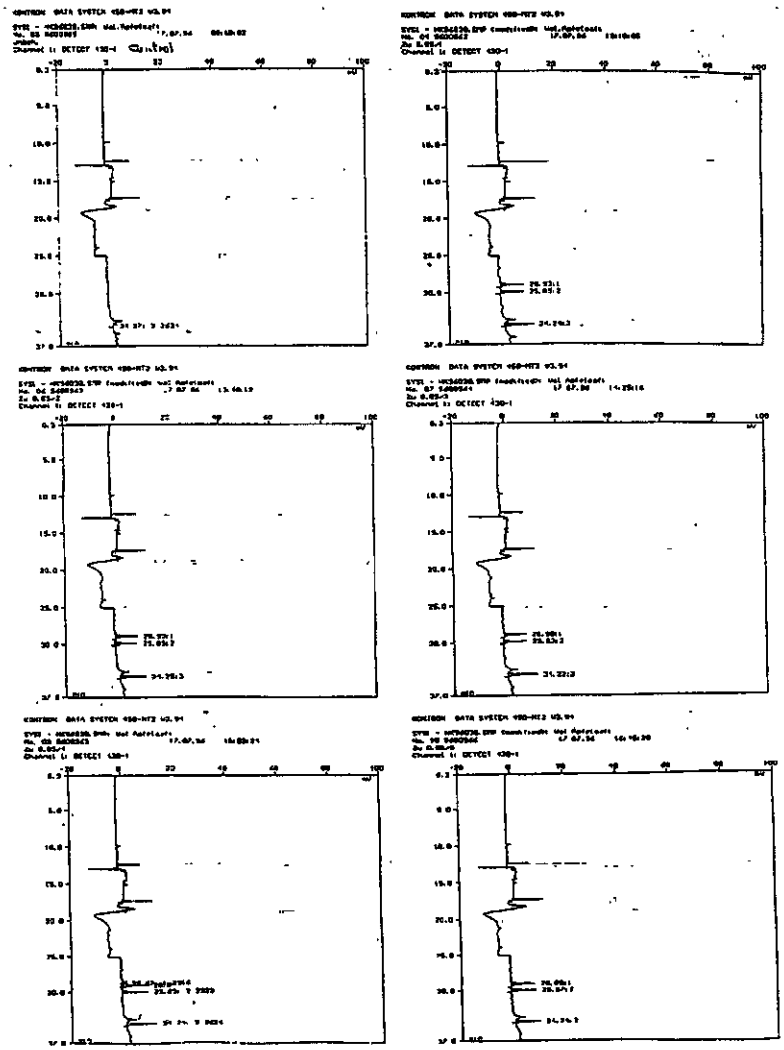


Figure 10 Method 350/3 in apple juice: Chromatogram of control and 5 fortified sample at 5.0 ppm.