INTRODUCTION

The Residue Analysis Unit (**henceforth named as RAU**) conducted a study to validate the standard operating procedures (coded C6^MAC, C0^MTF and C4^TSB) actually in force for the determination of **tetraconazole** residues in **drinking water, vegetal crops, and food of animal origin.**

Validation of the method was carried out according to the 91/414/EEC guidelines as reported in Annex VI and to the EC draft working document 8064/VI/97 (21/10/97).

Independent laboratory (**henceforth named as LAR**) validation was performed by:

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Analyses were done in accordance with protocol 2258 (see Enclosure B).

PROCEDURES

RECEPTION, STORAGE AND DELIVERY

Drinking water was directly drawn from the laboratory water supply and immediately fortified. Spiked samples were stored at +4 °C in the dark until analysis time.

Cereal (barley grain and straw) were obtained from ISAGRO RICERCA Biology and Agronomy Department, while **apples, tomatoes, milk, eggs, meat and fat** were bought directly on the market. Immediately after preparation and fortification, spiked samples were stored in the freezer deposit of the RAU, in the dark, below -20°C, according to the latest revised version of the Standard Operating Procedures (henceforth named SOPs) coded A1RIMO and A2CARI, until analysis time.

Grape (barbera, red bunches) were obtained from control plot of trial TIF/1/2I/V, prepared and fortified during the concomitant GLP study 2248. Spiked samples were stored in frozen conditions (-20°C) in the dark, as above, until analysis time.

Eventually **five** spiked samples for each fortification level plus **two** control (untreated) samples from each substrate were transferred from our testing facility to LAR.

All substrates were **delivered within 24 hours** from despatchment time.

Drinking water was sent at **ambient temperature**, while **all other substrates**, including the reference tetraconazole analytical standard, were sent **frozen** (in dry ice) in the dark to LAR.

IDENTIFICATION

All samples prepared were individually coded by RAU according to the SOP A3CODI.

LAR, upon reception, coded each sample according to their SOP: the correspondence to RAU codes is shown in Appendix in the individual certificates of analysis issued by LAR.

PREPARATION AND FORTIFICATION

Vegetal substrates, meat, fat and eggs

Apples and **tomatoes** were rinsed under tap running water. Afterwards each vegetal substrate was chopped and homogenized. **Meat** (bovine skeletal muscle) and **fat** (subcutaneous, bovine) samples were chopped and minced with a meat mincer. **Eggs** (from hens) were wipped (yolk and albumen together, without shell).

An exactly weighed amount of each substrate was transferred into several HDPE polyethylene flasks. Each flask was directly spiked with a known amount of tetraconazole analytical standard dissolved in acetone. After gentle whirling, the organic solvent was evaporated at room temperature with a light nitrogen stream. Eventually the whole homogenate was vigorously shaken. Spiked samples were stored until analysis time in the dark at -20 ° C.

Drinking water and milk

An exactly measured amount of tetraconazole analytical standard dissolved in acetone was poured into several HDPE polyethylene flasks. After evaporation of the organic solvent at room temperature with a light nitrogen stream, a measured amount of substrate was poured in each flask and vigorously shaken. Spiked samples were stored until analysis time in the dark (at + 4°C the drinking water ones, and at -20 ° C the milk ones).

ANALYTICAL METHOD

1. PRINCIPLE OF THE METHOD

Tetraconazole (see structural formula and nomenclature in Enclosure A) is extracted from the substrate with organic solvents (acetone and/or dichloromethane).

Final extracts are purified by column chromatography on alumina. The active ingredient is determined by gas chromatography using either a flameless ionization detector, calibrated

for nitrogen, or an electron capture detector (ECD).

2. REAGENTS

- Analytical RPE grade dichloromethane (e.g.: Carlo Erba - Milan Italy), redistilled in a glass apparatus.
- Analytical RPE grade hexane (e.g.: Carlo Erba - Milan Italy), redistilled in a glass apparatus.
- Analytical RPE grade acetone (e.g.: Carlo Erba - Milan Italy), redistilled in a glass apparatus.
- Analytical RS grade ethyl acetate (e.g.: Rudi Pont - Milan Italy), redistilled in a glass apparatus.
- Analytical RPE grade acetonitrile (e.g.: Carlo Erba - Milan Italy).
- n-hexane:acetone (50:50 v/v) mixture.
- Celite 209 (Celite Italiana S.r.l. Milan Italy) or Celite 545 (art.2093 Merck Darmstadt Germania).
- Liquid antifoaming agent (e.g.: DOW CORNING DB-100 (EU) BDH Milan Italy).
- Saturated aqueous solution of analytical grade sodium chloride (e.g.: Carlo Erba - Milan Italy).
- Alumina, grade II-III according to Brockman (e.g.: Merck - Milan Italy).
- Reference analytical standard of tetraconazole.
- Anhydrous sodium sulphate analytical RPE grade (e.g.: Carlo Erba - Milan Italy).
- Hydrogen gas for gas chromatography (SIO Novara Italy).
- Helium gas for gas chromatography (SIO Novara Italy).
- Nitrogen gas for gas chromatography (SIO Novara Italy).

3. EQUIPMENT

- Horizontal shaker for separatory funnel and conical flasks (e.g.: mod.HS501 digital IKA Milan Italy).

- Chopper and mincer (AVALLI mod.TR32 - Casabella Novara Italy or equivalent).
- Grinder homogenizer (BRAUN ZK100 MULTIMIX mod.4249 - Casabella Novara Italy or equivalent).
- Biotriturator for vegetal substrates (CARAVAGGI - Pontoglio Brescia-Italy or equivalent).
- Blade stainless steel homogenizer (SORVALL OMNI MIXER mod.17106 - OMNI INTERNATIONAL c/o ANALYTICAL CONTROL S.p.A. Cinisello Balsamo Milan Italy or equivalent).
- Witt apparatus with Büchner funnel.
- Extrelut™ extraction column or refill pack (Merck - Milan Italy or equivalent).
- Ultrasonic bath (e.g. mod. B3 Branson Milan Italy).
- Glass fiber paper filter (e.g. GF/C ϕ 9 cm and ϕ 4.7 cm, Whatman Milan Italy).
- Vacuum rotary evaporator (e.g.: LABO ROTA mod S300 Resona Technics Milan Italy).
- Gas chromatograph (e.g.: Mega Series II GC mod.8530 Thermo Quest Milan Italy) equipped with NPD-80 FL flameless detector and automatic peak integrator (e.g.: DP800 CH2 Carlo Erba Instruments Milan Italy).
- Gas chromatographic semicapillary column (e.g. SPB 5, length 30m, i.d. 0.53 mm, film thickness 1.5 µm - Supelco Milan Italy).
- 10 µL gas chromatographic syringe (e.g. Hamilton Milan Italy)
- technical balance (e.g. Sartorius mod. LC 820 - Zeiss Milan Italy)
- analytical balance (e.g. Sartorius mod. RC 250S - Zeiss Milan Italy)
- common analytical laboratory glassware and equipment for chemical laboratory.

4. METHOD

The method works out through these four steps:

PHASE 1: PREPARATION
PHASE 2: EXTRACTION

PHASE 3: PURIFICATION
PHASE 4: ANALYSIS

4.1 PHASE 1: PREPARATION

Depending on the substrate, this step can be omitted (liquid substrates, like water and milk) or specifically carried out by means of a mincer, a whipper, a mill or a biotriturator.

Aqueous vegetables (like tomatoes, grape bunches and apples), meat and fat are chopped in small pieces and subsequently homogenized in semi frozen conditions by means of a mincer above described in the equipment section).

Grain is milled by mechanical grinding (e.g.: with the Braun homogenizer ZK100 above described).

Straw is finely triturated and passed through a 3 mm hole stainless sieve using the biotriturator above said.

Eggs, deprived from the shells, are simply whipped.

A representative portion of each commodity (about 1 kg) must be processed and prepared as above described. Draw at least 200 g and store at about -20°C until extraction time.

4.2 PHASE 2: EXTRACTION

Vegetal crops.

Homogenize 30 g (10 g for straw) of prepared sample with 100 mL acetone in the Omni Mixer for 15 min at about 3000 r.p.m.

Filter the mixture under vacuum through the Büchner funnel using a filter disk GF/C, wash the container and the cake twice with 100 mL acetone, collect extracts in a 500 mL round bottomed flask and evaporate under vacuum with the rotary evaporator until all acetone has been removed (beware of tumultuous boiling, keep temperature below 40°C).

Transfer the aqueous residue in a 250 mL separatory funnel, adding 80 mL of water and extract with 100 mL dichloromethane shaking for at least 5 minutes. Add 25 mL saturated aqueous solution of sodium chloride and let phases to separate.

Filter the organic phase through 20 g anhydrous sodium sulphate in a 250 mL round

bottomed flask and evaporate the solvent as above mentioned.

Meat and fat.

Draw an amount (e.g.: 30 g), add acetone (100 ml), mix as much as possible with a glass rod and homogenize with the mixer Sorvall at 300 rpm for 15 min at least. Add 10 g Celite in fat samples only.

Filter through GF/C under vacuum in a round bottomed flask (500 ml) and evaporate the organic solvent, adding an antifoam agent. Fat leaves a dry residue, while other tissues (skeletal muscle, kidney etc.) leave a slurry that contains water. For this latter substrates it is necessary to extract with dichloromethane in a separatory funnel the aqueous residue (10 min at 200 strokes/min): add 50 ml saturated NaCl solution, shake again for 1 min and leave the phase to separate for at least 1 hour. Filter through Na_2S0_4 (5 cm x 1 cm diameter) in a 250 ml round bottomed flask and evaporate all the organic solvent with the rotary evaporator under vacuum until a dry residue is obtained.

Dissolve the dry residue with n-hexane (100 ml) and extract with acetonitrile (use saturated solvents). Collect the acetonitrile phase and evaporate again to dryness.

Eggs and milk.

Pour 20 g (25 g for milk) on the top of an Extrelut™ column. When the liquid has been completely adsorbed, wait at least for 15 min until the aqueous phase is uniformly distributed in the column filling; this operation step can be facilitated, if necessary, by means of a slight suction from the bottom of the column. Add 75 ml of n-hexane and collect the eluate (about 60 ml) in a round bottomed flask. Evaporate the solvent as above mentioned.

Water

Shake in a glass separatory funnel equipped with a teflon stopcock 1 liter of water with 100 ml methylene chloride trice (or 100 ml water with 50 ml methylene chloride twice or 10 ml water with 5 ml methylene chloride twice, depending on the presumable content of active ingredient) for 15 min.

Filter the organic phase through 30 g anhydrous sodium sulphate previously washed with methylene chloride (50 g for organic volumes from 300 to 100 ml, 10 g for smaller volumes). Wash once the NA_2SO_4 with methylene chloride (1 ml for each gram employed).

Collect the filtrate in a round bottomed flask of proper size and evaporate the solvent under reduced pressure in a rotavapor (max temp. 30 °C).

4.3 PHASE 3: PURIFICATION

4.3.1 Column preparation

Pour 15 mL of the n-hexane : acetone mixture into the chromatographic tube. Slowly add 20 g alumina (free from air bubbles). Allow to settle then add 5 g anhydrous sodium sulphate. Drain the solvent and wash with further 40 mL of the same mixture, at a flow rate of 1.5 mL/min until the liquid cover the top of the filling.

4.3.2 Extract loading

Dissolve the residue obtained after solvent evaporation (see extraction paragraph, above) with 2 mL of the same n-hexane:acetone mixture, using the sonicating bath, if necessary.

Transfer quantitatively into the column so prepared, leaving the liquid to penetrate into the filling.

Wash twice the flask with 1 mL of the same solvent mixture and top to the column.

4.3.3 Chromatographic elution

Eluate with the same solvent mixture: discard the first 40 mL fraction and collect the following 60 mL fraction.

Evaporate the solvent to dryness as above mentioned. Dissolve the residue in a suitable volume of ethyl acetate (from 1 up to 50 mL, according to the hypothetical concentration of tetraconazole).

4.4 PHASE 4: ANALYSIS

Dissolve the residue in an appropriate volume of ethyl acetate and analyse by gas chromatography using either a nitrogen phosphorous detector set for nitrogen (GC/NPD) or an electron capture detector (GC/ECD). This latter equipment is advisable for all biological substrates.

The operative conditions used and the integration parameters are shown in the gas chromatographic section.

5. CALCULATIONS.

Calculation of tetraconazole is accomplished by the external standard method, on at least duplicate injections.

Exactly measured volumes of a tetraconazole reference standard solution at known concentration (C_{std}) are injected just before and after sample analysis.

Peak height of treated sample (H_{smp}), of blank (untreated) sample (H_{bkg}), and of standard (H_{std}) are recorded.

The concentration of tetraconazole present in the original sampled substrate is then calculated by applying the formula below:

$$R = \frac{(H_{smp} - H_{bkg}) * C_{std} * V_F}{H_{std} * W}$$

where:

R residue (in mg/kg or mg/L).

H_{smp} unknown treated sample peak height (arbitrary units: datum is the mean of at least duplicate injections)

H_{bkg} unknown blank (untreated) sample peak height (arbitrary units: datum is the mean of at least duplicate injections)

C_{std} reference standard concentration (mg/L)

V_F final purified volume (mL)

H_{std} reference standard peak height (arbitrary units: datum is the mean of at least duplicate injections)

W amount of the sample (g or mL)

The subtraction of H_{bkg} from H_{smp} in the equation above shown, is performed (for procedural recovery samples only) if H_{bkg} is higher than 10% of H_{smp}.

The time necessary to perform a complete analysis (including data elaboration) is about 2.5 hours/man for all substrates.

6. IMPORTANT POINTS

The recovery factor of the method may be strongly affected by interfering substances; as a consequence it is necessary to perform always recovery tests at different fortification levels.

For the same reason it could be necessary to modify either the ratio of the solvent mixture used for the clean-up step or the operative conditions of analysis.

Moreover, different batches of alumina may require different elution conditions: for this reason it is necessary to check the recovery of tetraconazole from the purification column for every new lot of the stationary phase.

Beware of tumultuous boiling at the beginning of solvent evaporation.

Sonicate solutions whenever rinsing and washing operations are carried out.

METHOD VALIDATION

LINEARITY

The linearity of responses for **tetraconazole** has been checked at RAU over the range 0.08÷8 mg/L and at LAR over the range 0.5÷1 mg/L. The performance of the gaschromatograph was checked before and after the series of analyzed samples.

CALIBRATION

Recovery tests have been performed on untreated samples fortified **with tetraconazole at different concentrations**, according to the SOP B3^FST.

Each fortification level was analyzed in **quintuplicate**, starting from the raw fortified substrate, both at RAU and at LAR. **Control** samples (untreated substrate) have been analysed in parallel in **duplicate**.

PRECISION

According to AOAC Guidelines for collaborative Study procedure (1990) and ISO Guide 18, ISO5725-1986 definition, r values (repeatability) have been calculated according to the formula:

$$r=2*\sqrt{2}*X*\frac{RSD}{100}=2*\sqrt{2}*SD$$

where X is the arithmetic mean, RSD and SD are the relative and the absolute standard deviation of the population of recovery data (n-1) obtained from repeated analysis of the same substrate performed at RAU by the same operator with the same equipment using the same method.

Similarly R values (reproducibility) have been calculated according to the formula:

$$R=2*\sqrt{2}*X*\frac{RSD}{100}=2*\sqrt{2}*SD$$

where X is the arithmetic mean, RSD and SD are the relative and the absolute standard deviation of the population of recovery data (n-1) obtained from repeated analysis of the same substrate performed at a different laboratory (LAR) by the same operator with the same equipment using the same method.

LIMITS OF DETECTION AND OF DETERMINATION

The limit of quantitative determination (LOQ) and the limit of detection (LOD) have been established after statistical analysis of the recovery data at the lowest fortification level and of control samples.

SAMPLE ANALYSIS

Analyses were performed according to the method above described.

The weight of each sample undergone to the analytical procedure, both at RAU and at LAR, was **30 g** for vegetal crops, meat and fat tissues (only exception the **straw**, where **10 g** were extracted), **25 g** for milk, **20 g** for eggs and **1 L** for water.

All samples (both final extracts and working standard solutions) were analysed for **tetraconazole** content by double injection (2 µL). Analytical reference standard was injected at least every three samples (i.e. every series of six injections, corresponding to Δt~60 min), according to the sequence:

... ➡ ext.std (1st inj.) ➡ ext.std (2nd inj.) ➡ sample 1 (1st inj.) ➡ sample 1 (2nd inj.) ➡ sample 2 (1st inj.) ➡ sample 2 (2nd inj.) ➡ sample 3 (1st inj.) ➡ sample 3 (2nd inj.) ➡ ...

Peak heights were automatically integrated and the calculation of the concentration of each injected sample was performed by computerized elaborator according to the external standard method (see formula in the method above). The mean height of each couple of reference external standard (ext.std) was used for each calculation of the following series of six injections.

The chronological complete sequences of injections as well as the volume (in mL) of the final purified extract and amount (in g) of sample analysed by NPD at RAU are shown in Tables 27 to 31, (see column headings XF and S.A., respectively for volumes and sample amounts). The concentrations (in ppm) calculated for each single injection are listed in Tables 32 to 36.

Similarly the chronological complete sequence of injections, volumes (in mL) of the final purified extracts, amount of sample analysed by ECD at RAU and the concentrations (in ppb) calculated for each single injection are reported in Table 37.

Note that the values reported are merely the data obtained by applying the formula for the calculation of residue (see method in the text) to the automatically integrated peak. As a consequence they do not take in account of the established qualitative and quantitative limits validated for the analytical procedure.

Final data were eventually obtained by automatic averaging the concentrations of each couple of injections.

STABILITY DATA

The stability of **tetraconazole** in several vegetable aqueous substrates, stored **in the dark, below -20°C,** according to EPA Guideline CRC 737-R-93-01 January 1993 and to SOP B3^FST, has been checked at this facility.

The product remained substantially unchanged after about three years storage.

STANDARD

Tetraconazole analytical standard used in this study was supplied by ISAGRO RICERCA with its certificate of analysis (Enclosure A) and was stored in freezer at about -10°C.

Tetraconazole working standard solutions were prepared by dissolving an amount (about 100 mg) of the analytical standard in ethyl acetate, accurately weighed according to the SOPs coded A0BIL1.

GRAPHICAL SOFTWARE

All the graphical elaborations of data have been carried out using the Sigma Plot® Scientific graphing software version 4 - Jandel Europe Schimmelbuschstr 25, 40699 Erkrath (Germany).

tetraconazole

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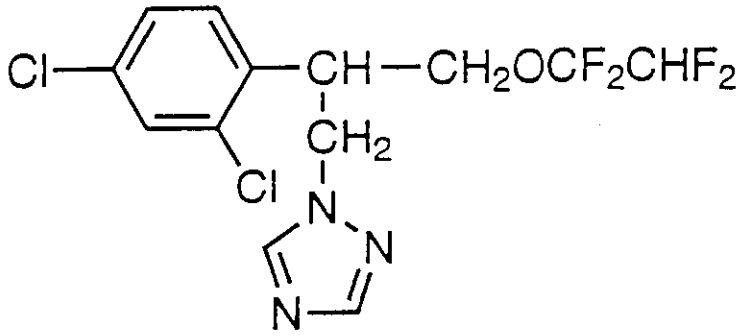

NOMENCLATURE

Common name tetraconazole (BSI, draft E-ISO).

IUPAC name (*RS*)-2-(2,4-dichlorophenyl)-3-(1*H*-1,2,4-triazol-1-yl)propyl 1,1,2,2-tetrafluoroethyl ether.

C.A. name (±)-1-[2-(2,4-dichlorophenyl)-3-(1,1,2,2-tetrafluoroethoxy)propyl]-1*H*-1,2,4-triazole. **CAS RN** *[112281-77-3]* unstated stereochemistry

Development code M 14 360 (Agrimont).