Dossier According to Directive 91/414/EEC

TERPENOID BLEND (A-TERPINENE, P-CYMENE, D-LIMONENE) QRD 460

Active substance for insect pest control developed from plant extract of *Chenopodium ambrosioides* near *ambrosioides*

DOCUMENT MII, Section 3

TOXICOLOGICAL AND TOXICOKINETIC STUDIES ON THE ACTIVE SUBSTANCE

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5 TOXICOLOGICAL STUDIES

Terpenoid Blend (α-terpinene, p-cymene, and d-limonene) QRD 460 is a new active substance developed by AgraQuest Inc. based originally on the naturally occurring extract of the plant species *Chenopodium ambrosioides* near *ambrosioides* for use as an insecticide plant protection product.

To defend themselves against herbivores and pathogens, plants naturally release a variety of volatiles including various alcohols, terpenes and aromatic compounds. These volatiles can deter insects or other herbivores from feeding, can have direct toxic effects on pests, or they may be involved in recruiting predators and parasitoids in response to feeding damage (Ashour *et al*. 2010). They may also be used by the plants to attract pollinators, protect plants from disease, or they may be involved in interplant communication. As these properties have been known and observed for a very long time, it is a natural progression that three such terpenes, α-terpinene, p-cymene, and d-limonene, have been identified as candidates for biopesticidal use. In the original plant extract the three terpene compounds in combination are the source of insecticidal activity: as this naturally occurring combination is the key active moiety, they are considered and termed to be one active substance. This consideration was agreed at the DG SANCO Phytopharmaceutical Standing Committee meeting 26-27 November 2009 for QRD 420, which contains the same active substance as QRD 460.

The original plant extract (QRD 406) was registered by US EPA as a biopesticide in April 2008. The initial active substance and product was based on a plant extract of *Chenopodium ambrosioides* near *ambrosioides*. The essential oil was harvested from the plant biomass using steam distillation. Variability in growing conditions for the plants meant this active substance suffered from variability in the concentration of the three constituent active terpenes and so an alternative, QRD 460 was developed which is an optimized blend of the three terpenes that reflects the proportions found in the original plant extract QRD 406.

AgraQuest Inc. has submitted this application for approval of the new active substance QRD 460 and its product, QRD 452 respectively, for registration in the EU with ctgb Netherlands as the Rapporteur Member State. It is an insecticide for use on tomatoes and peppers in glasshouses and cucurbits in glasshouses and field at a maximum application rate of 1.523 kg a.s./ha up to 3 times with a 7 day interval between treatments.

Table 6-1: EU Critical GAP for QRD 460 use on Tomatoes, Peppers and Cucurbits

Region	Outdoor/ Protected	Max. No. of Applications	Application Interval (days)	Max. Application		Minimum PHI (days)
				Rate (kg as/ha)	Water (L/ha)	
N EU	Protected	3	7	0.381 – 1.523	400 - 1000	0
S EU	Protected	3	7	0.381 – 1.523	400 - 1000	0
S EU	Outdoor	3	7	0.762 – 1.523	400 - 1000	0

The mode of action of the product is considered non-toxic. Based on laboratory and field trial observations, the mechanism for controlling insect pests is considered to be through degradation of soft insect cuticles resulting in a disruption of insect mobility and respiration. This is considered to occur by direct contact and localized fumigant action. For further details, please refer to document MIII, Section 7, Point 6.

It is noteworthy that these terpenes, α-terpinene, p-cymene, and d-limonene, are commonly used as fragrances and flavourings (Joint FAO/WHO Expert Committee on Food Additives & WHO Technical Report Series 928.). They are present in abundance in many herb plants, and are common in many other edible plants such as citrus fruits, tomatoes, celery and carrots, with various functions as secondary metabolites (Ashour, *et al*, (2010)). Consequently they are a ubiquitous part of both human and animals' natural diet and it is reasonable to expect regular contact with them in the environment without any concern.

All three terpenes are also found, to a greater or lesser extent, in the following EU registered or pending active substances: tea tree oil, thyme oil, orange oil, citronella, spearmint oil, and tagetes (marigold) oil.

Due to the chemical nature of the three terpenes in QRD 460, they disperse rapidly via volatilisation and leave little to no residues (see Section 4 Metabolism and Residues). Equally they disperse rapidly in the environment into the air and then degrade (see Section 5 Environmental Fate) and so any possible toxicological exposure is expected to be

minimal. Additionally, the three terpenes are naturally occurring, are ubiquitous and normal exposure presents no significant risk to humans, animals or the environment, so the plant protection use proposed here adds nothing of significance to the natural exposure, so no additional data other than what is presented here is considered necessary.

The components of the active substance have high vapor pressures and high Henry's Law Constants which means the active substance is highly volatile and evaporates quickly. In addition, it has been shown that the active substance does not persist in the environment. Because QRD 460/452 dissipates so quickly from the sprayed plant surface, as well as the soil, water, and air, each application is in effect a single acute event.

It is reasonable to conclude that even repeat applications may each be considered as single acute events rather than as chronic exposures. In addition, should exposure occur the active substance components have been shown to be rapidly metabolized and excreted in mammalian systems.

The studies presented here under Section 3 Toxicology demonstrate that there are no significant toxicological concerns with regard to the plant protection use of QRD 460 and its product QRD 452 presented here for registration.

To aid evaluation of the dossier, the code designations are described so that it is clear which test substance was used for each study. All substances listed are considered substantially equivalent.

Code Designations
The various AgraQuest code designations that relate to the active substance, products and the submitted documents are as follows:

QRD 406 = *Chenopodium ambrosioides* near *ambrosioides* plant extract technical grade active ingredient (tgai) – consisting of the three terpenes as the active component plus plant derived impurities. Three terpenes comprise approximately 68% of QRD 406.

QRD 400 = formulated EC product with 25% plant extract (QRD 406) active ingredient, 75% other formulants (Also known as FACIN 25EC in some reports and registered in the USA as Requiem® 25EC and Metronome™.) The three terpenes in QRD 400 comprise approximately 17%.

QRD 420 = blended tgai using the three terpenes in the same concentrations as found in QRD 406 with plant derived impurities replaced with canola oil. The three terpenes comprise approximately 67% of QRD 420.

QRD 416 = formulated EC product with 25% blended (QRD 420) a.i., 75% other formulants (same formulants in the same concentrations as QRD 400). The three terpenes comprise approximately 16.75% (w/w) of QRD 416.

QRD 452 = QRD 416 – due to a code designation error, the product was re-coded as QRD 452. There are a few studies that reference QRD 416, but the composition is identical to QRD 452. (Also known and registered in the USA as Requiem® EC and Metronome™ EC). The concentration of the three terpenes in QRD 416 and QRD 452 is 16.75%.

QRD 460 = Blended tgai without canola oil. This contains only the three terpenes. The proportions of the three terpenes are essentially the same as the plant extract tgai minus plant derived impurities. So, less QRD 460 is required in Requiem® EC (QRD 452), 16.75% instead of 25%. The percentage of each terpene in QRD 452 and QRD 400 are the same.

IIA 5.1 Absorption, distribution, excretion and metabolism in mammals

The terpenes in QRD 460 are commonly occurring compounds found in many plants, there is extensive data and publicly available literature for these components, so no data are provided specifically for QRD 460. The components of QRD 460, α-terpinene, p-cymene, and d-limonene are the most common terpenes in nature; WHO[1] report that being lipophilic, alicyclic hydrocarbons such as α-terpinene and d-limonene are likely to use passive diffusion to

[1] WHO (2005), Evaluation of Certain Food Additives. WHO Technical Report Series No. 928. 63rd Report of the Joint FAO/WHO Expert Committee on Food Additives.

cross biological membranes. After inhalation or oral exposure they are rapidly absorbed and distributed then elimination from blood is triphasic with a slow terminal phase. All these flavouring agents are considered to have similar pathways of metabolism in animals and humans. Post absorption they are oxidised via cytochrome P450 enzymes and alcohol/aldehyde dehydrogenases into polar oxygenated metabolites. Alicyclic substances like α-terpinene and d-limonene are oxidised by side chain oxidation or by epoxidation of the exocyclic or endocyclic double bond. The hydroxylated metabolites yielded are excreted in conjugated form or are subject to further oxidation to yield more polar metabolites which are rapidly excreted in the urine. Where a double bond is present, epoxide metabolites may be formed and these metabolites are further detoxified by hydrolysis or by conjugation with glutathione. Joint FAO/WHO Expert Committee on Food Additives (JECFA) considered flavouring agents such as these would be expected to be metabolised to innocuous products.

The above data are supported by data published by IPCS in 1998[2] and IARC[3]; most data are available for d-limonene. Falk *et a*l (1990)[4] reported d-limonene has a high partition coefficient between blood and air ($\lambda_{blood/air}$ = 42) and is readily taken up in the blood via the alveoli, the net uptake of d-limonene in human volunteers exposed at 450, 225, and 10 mg/m^3 for 2 hours during light physical exercise averaged 65%[5]. Igimi et al., 1974[6] and Kodama et al., 1976[7] showed d-limonene administered via the oral route is rapidly and almost completely taken up from the gastrointestinal tract in humans and animals and they excreted 52-83% of the dose in their urine within 48 hours. Distribution around the body is rapid and it is readily metabolised; clearance from the blood was 1.1 litre/kg bodyweight per hour in human males exposed at 450 mg/m^3 for 2 hours[4]. The same authors suggest a high affinity to adipose tissues is indicated by a high oil/blood partition coefficient and a long half-life during a slow elimination phase[3,4]. Igimi et al., 1974[5] found that after oral administration of radiolabled [^{14}C] d-limonene to male rats, the distribution of radioactivity was initially high in the liver (maximal after 1 hour), kidneys (maximal after 2 hours), and blood (maximal after 2 hours) but negligible amounts were detected after 48 hours. It is recognised that in male rats, d-limonene exposure causes damage to the kidneys and renal tumours; indeed the concentration of d-limonene equivalents was approximately 3 times higher in male rats than in females, 40% of which was reversibly bound to the male rat specific protein, alpha2μ-globulin[8],[9]. Because this protein is male rat specific, these data are considered not to be relevant for human risk assessment.

IPCS[2] report the biotransformation of d-limonene has been studied in many species, with some differences noted between species with respect to the metabolites present in both plasma and urine. Kodama et al[7] reported urinary recovery of d-limonene was 77-96% within 3 days in rats, guinea-pigs, hamsters and dogs, with faecal recovery of 2-9% within 3 days; indeed for all species most excretion occurred within the first 24 hours. Following an oral dose of d-limonene in humans, approximately 25-30% was identified in urine as d-limonene-8,9-diol and its glucuronide; approximately 7-11% was eliminated as perillic acid (4-(1-methylethenyl)-1-cyclohexene-1-carboxylic acid) and its

2 IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene

3 IARC Monographs (1995). D-Limonene Volume 73.

4 Falk A, Gullstrand E, Löf A, Wigaeus Hjelm E (1990) Liquid/air partition coefficients of four terpenes. British journal of industrial medicine, 47:62-64. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

5 Falk Filipsson A, Löf A, Hagberg M, Wigaeus Hjelm E, Wang Z (1993). d-Limonene exposure to humans by inhalation: Uptake, distribution, elimination, and effects on the pulmonary function. Journal of toxicology and environmental health, 38:77-88. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

6 Igimi H, Nishimura M, Kodama R, Ide H (1974) Studies on the metabolism of d-limonene (p-mentha-1,8 diene). I. The absorption, distribution and excretion of d-limonene in rats. Xenobiotica, 4:77-84. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene and IARC Monographs (1995). D-Limonene Volume 73.)

7 Kodama R, Yano T, Furukawa K, Noda K, Ide H (1976) Studies on the metabolism of d-limonene (p-mentha-1,8-diene). IV. Isolation and characterization of new metabolites and species differences in metabolism. Xenobiotica, 6:377-389. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene) and IARC Monographs (1995). D-Limonene Volume 73)

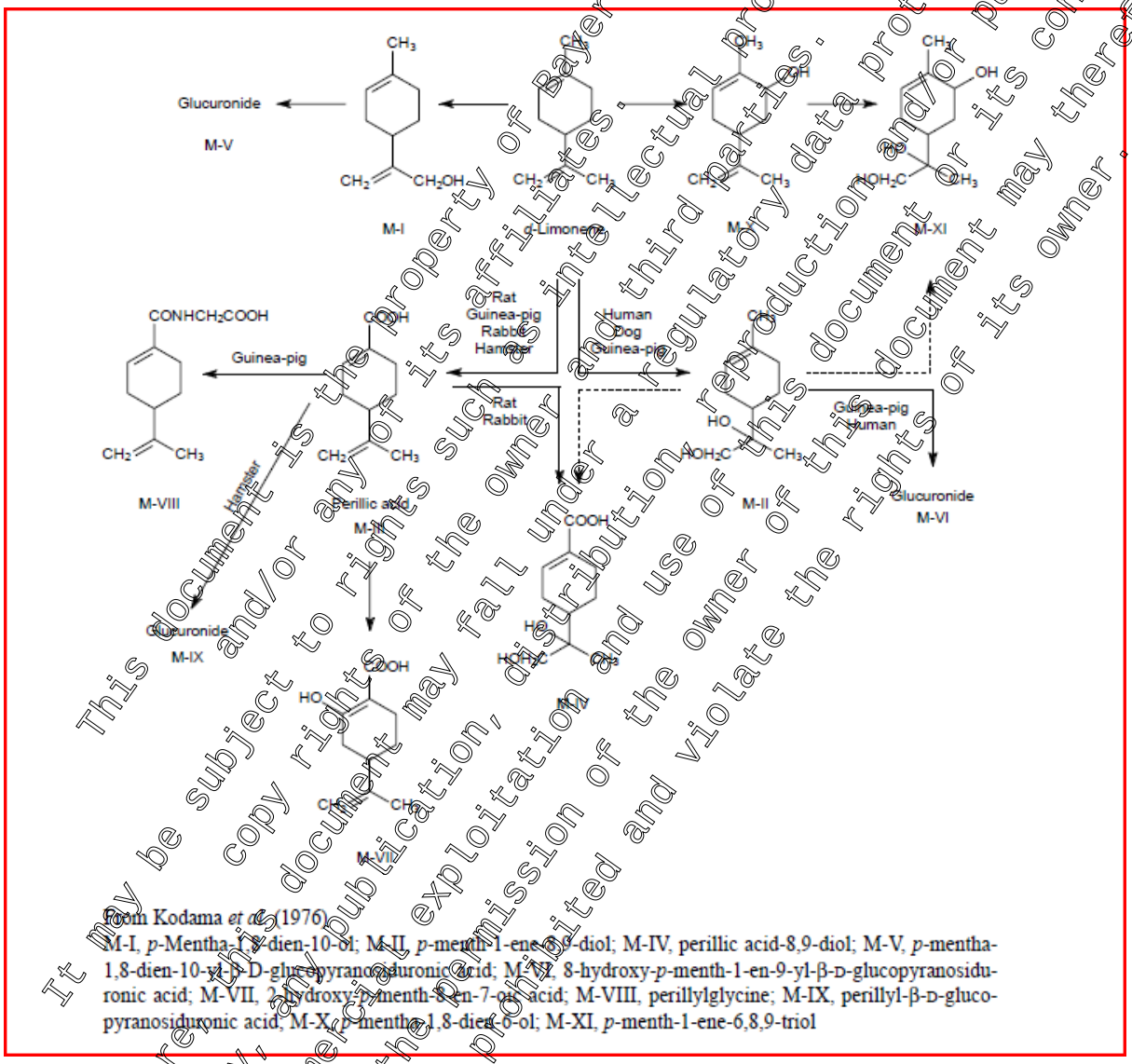
8 Lehman-McKeeman LD, Rodriguez PA, Takigiku R, Caudill D, Fey ML (1989). d-Limonene induced male rat specific nephrotoxicity: Evaluation of the association between d-limonene and alpha2μ-globulin. Toxicology and applied pharmacology, 99:250-259. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

9 Lehman-McKeeman LD, Caudill D (1992) Biochemical basis for mouse resistance to hyaline droplet nephropathy: lack of relevance of the alpha2μ-globulin protein superfamily in this male rat specific syndrome. Toxicology and applied pharmacology, 112:214-221. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

metabolites[7,10],. However Crowell *et al*., (1992) report perillic acid to be the principal metabolite in the plasma of humans and rats[11].

Falk Filipsson *et a*l., (1993)[4] reported three phases of elimination were observed in the blood following inhalation exposure of volunteers to d-limonene at 450 mg/m^3 for 2 hours; half-lives of 3, 33, and 75 minutes were recorded. Approximately 1% of that taken up was eliminated unchanged in exhaled air, whereas and 0.003% was eliminated unchanged in the urine.

The possible metabolic pathway for limonene (reproduced from IPCS[2] and WHO[1], original citation from Kodama *et al*, 1976[7].).



From Kodama *et al*, (1976)
M-I, *p*-Mentha-1,8-dien-10-ol; M-II, *p*-menth-1-ene-6,9-diol; M-IV, perillic acid-8,9-diol; M-V, *p*-mentha-1,8-dien-10-yl-β-D-glucopyranosiduronic acid; M-VI, 8-hydroxy-*p*-menth-1-en-9-yl-β-D-glucopyranosiduronic acid; M-VII, 2-hydroxy-*p*-menth-8-en-7-oic acid; M-VIII, perillylglycine; M-IX, perillyl-β-D-glucopyranosiduronic acid; M-X, *p*-mentha-1,8-dien-6-ol; M-XI, *p*-menth-1-ene-6,8,9-triol

[10] Smith QW, Wade AP, Dean FM (1969) Uroterpenol, a pettenkofer chromogen of dietary origin and a common constituent of human urine. Journal of endocrinology, 45:17-28. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

[11] Crowell PL, Elegbede JA, Elson CE, Lin S, Vedejs E, Cunningham D, Bailey HH, Gould MN (1992) Human metabolism of orally administered d-limonene. Proceedings of the American Association for Cancer Research, 33:524. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

When Regan *et al* (1976) administered limonene orally to rats, ten terpinoid metabolites were identified in urine; of these 7 were identified as p-mentha-2,8-dien-l-a-ol, p-mentha-2,8-dien-l-/3-ol, p-mentha-1,8-dien-6-a-ol, p-mentha-1,8-dien-6-0-ol, p-mentha-2-ene-8,9-diol, p-mentha-1,8-dien-7-ol, and 4-isopropenyl-l-cyclohexene-l-carboxylic acid[12].

p-Cymene, as a lipophilic aromatic hydrocarbon, is also very likely to cross biological membranes by passive diffusion. WHO reported the available data on p-cymene indicate it is readily absorbed from the gastro-intestinal tract, distributed widely and metabolised and excreted mainly in urine. WHO anticipate p-cymene will have a similar pathway of metabolism in animals and humans. Post absorption it is oxidised via cytochrome P450 enzymes and alcohol/aldehyde dehydrogenases into polar oxygenated metabolites. The principle metabolic pathway of aromatic terpene hydrocarbons involves hepatic microsomal cytochrome P450 oxidation of the side-ring chains to yield alcohols, aldehydes and acids. The metabolites are then conjugated with glycine, glucuronic acid or glutathione and excreted in the urine and bile. The committee considered p-cymene would be expected to be metabolised to innocuous products. This is supported by the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in contact with Food (AFC) who report p-cymene is oxidised at the isopropyl side chain yielding 2-(p-tolyl)-2-propanol which is not oxidised further, but is excreted unchanged or as a glucuronic conjugate[13].

Pass *et al* (2002) studied the metabolism of p-cymene in the common brushtail possum, the koala and the rat[14]. The chemical is found in the leaves of the eucalyptus and is regularly ingested in the diet of the possum and koala. The major metabolite in all species was cuminyl alcohol with lesser amounts of other side-chain alcohols, no phenolic metabolites were detected. Pre-treatment with a terpene rich diet increased the cuminyl alcohol formation in possums indicating that a terpene diet induces enzymes responsible for its metabolism. The authors ranked the different species in their ability to metabolise the material: terpine-treated possum>control possum>koala>rat.

Matsumoto *et al* (1992)[15] reported p-cymene is metabolised in rabbits to four optically active metabolites, 2-(p-tolyl)-1-propanol, 2-(p-tolyl)propanoic acid, p-(2-hydroxy-1-methylethyl)benzoic acid and p-(1-carboxyethyl)benzoic acid, also three optically inactive metabolites, 2-(p-tolyl)-2-propanol, p-isopropylbenzoic acid, and p-(1-hydroxy-1-methylethyl)benzoic acid. Walde *et al*[16] (1983) studied the metabolism of p-cymene in Wistar male rats or female Dunkin Hartley guinea pigs following oral or inhalation administration of 100 mg/kg. 80 and 71% respectively was extracted in the urine as extractable metabolites, the authors speculated the rest was excreted in the faeces or as unextractable metabolites in urine; excretion was nearly complete within 48 h. In total 18 urinary metabolites were detected and identified, these comprised monohydric alcohols, diols, mono- and di-carboxylic acids and hydroxyacids; rats did not excrete two of the 18 metabolites and guinea-pigs did not excrete a third. Boyle et al studied the metabolism of p-cymene in rats following an oral dose of 50 and 200 mg/kg. Again metabolism occurred within 48 hours in urine; the metabolites were 2-p-toly-propanol (39% of the recovered dose) and 2-p-carboxyphenylpropan-2-ol (19%) with minor metabolites 2-p-carboxyphenylpropan-1-ol (10%), 2-p-carboxyphenylpropanoic acid (14%) and p-isopropylbenzioc acid (17%)[17].

In summary, whilst there are no ADME data for QRD 460, published data exist for its terpene components d-limonene and p-cymene and these data indicate the terpenes have similar pathways of metabolism in animals and humans. It is also reasonable to assume that α-terpinene will be metabolized in essentially the same manner as d-

[12] Regan JW and Bjeldanes LF (1976). Metabolism of (+)-Limonene in rats. J Agric Food Chem Vol 24 377-380.

[13] Opinion of the Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC) related to Flavouring Group Evaluation 18 (FGE.18): Aliphatic, alicyclic and aromatic saturated and unsaturated tertiary alcohols, aromatic tertiary alcohols and their esters from chemical group 6 (2006). The EFSA Journal 331, 1-77.

[14] Pass GJ, McClean S, Stupans I and Davies NW (2002). Microsomal metabolism and enzymatic kinetics of the terpene p-cymene in the common brushtail possum (Trichosurus vulpecula), koala (Phascolarctos cinereus) and rat. Xenobiotica 32 (5) 383-397.

[15] Matsumoto T, Ishida T, Yoshida T, Terao H, Takeda Y, Asakawa Y (1992). The enantioselective metabolism of p-cymene in rabbits. Chem Pharm Bull (Tokyo). Jul;40(7):1721-6.

[16] Walde A, Ve B, Scheline RR, Monge P (1983). p-Cymene metabolism in rats and guinea-pigs. Xenobiotica.. Aug;13(8):503-12. Cited in Opinion of the Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC) related to Flavouring Group Evaluation 18 (FGE.18): Aliphatic, alicyclic and aromatic saturated and unsaturated tertiary alcohols, aromatic tertiary alcohols and their esters from chemical group 6 (2006). The EFSA Journal 331, 1-77.

[17] Boyle R et al. (1999). Comparaitive metabolism of dietary terpene, p-cymene, in generalist and specialist folivorous marsupials. J Chem Ecol 25, 2109-2126. Cited in Opinion of the Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC) related to Flavouring Group Evaluation 18 (FGE.18): Aliphatic, alicyclic and aromatic saturated and unsaturated tertiary alcohols, aromatic tertiary alcohols and their esters from chemical group 6 (2006). The EFSA Journal 331, 1-77.

limonene and p-cymene. Following an oral dose, the components of QRD 460 are rapidly and well absorbed from the gastrointestinal tract and metabolites are excreted, mostly via urine, within 48 hours (with the major part excreted within 24 hours). The amount of d-limonene absorbed via the oral route is similar in different species; reported values range from 50-96% in rats, guinea-pigs, hamsters and dogs whilst those in human male volunteers are reported as 50-80% (Kodama *et al*, 1976; Igimi *et al*., 1974). Absorption via the inhalation route is also rapid; the percentage absorbed is reported by Falk *et al* 1990 to average 65%. Similar absorption values are reported for p-cymene (70-80%) in rats and guinea pigs with recovery within 48 hours. Given the similar structure and properties of α-terpinene, absorption values are likely to be comparable. The available data indicate the components of QRD 460 are readily metabolised to materials which are rapidly excreted within 48 hours.

IIA 5.1.1 Toxicokinetic studies – single dose, oral route, in rats

The terpenes in QRD 460 are commonly occurring compounds found in many plants, there is extensive data and publicly available literature for these components, so no data are provided specifically for QRD 460: the data available are summarised in IIA 5.1 above.

IIA 5.1.2 Toxicokinetic studies – second single dose, oral route, in rats

The terpenes in QRD 460 are commonly occurring compounds found in many plants, there is extensive data and publicly available literature for these components, so no data are provided specifically for QRD 460: the data available are summarised in IIA 5.1 above.

IIA 5.1.3 Toxicokinetic studies – repeated dose, oral route, in rats

The terpenes in QRD 460 are commonly occurring compounds found in many plants, there is extensive data and publicly available literature for these components, so no data are provided specifically for QRD 460: the data available are summarised in IIA 5.1 above.

IIA 5.2 Acute toxicity

Acute toxicity studies have been conducted with QRD 460 and QRD 420. QRD 420 is very similar to QRD 460 containing the three terpenes in the same proportions but with the addition of canola oil (full composition details are provided in Document J as this information is confidential) and hence results obtained with QRD 420 are also valid for QRD 460. QRD 460 is harmful if swallowed; it is of low acute toxicity by the dermal and inhalation routes. It is not irritating to skin or eyes. QRD 460 is a moderate skin sensitiser under the conditions of the local lymph node assay.

While QRD 460 did give a positive result in the LLNA test, it is important to consider that two other tests conducted with substantially similar active substances using the Buehler method and the Magnusson and Kligman maximization method were negative for sensitization. In addition, the plant extract-based and terpenoid blend active substances have been manufactured for a number of years without a single report of dermal sensitization from manufacturing personnel. Similarly, the plant extract-based and QRD 452 plant protection products have been widely used (development trials and commercial use) in the USA with no reports of dermal sensitization or other adverse effects.

According to Commission Directive 67/548/EEC (as amended), classification and labelling as Xn; R22 Harmful if swallowed and R43 May cause sensitisation by skin contact are required. According to Regulation (EC) No.1272/2008 this means classification and labelling as Cat. 4 H302 Harmful if swallowed and Cat. 1 H317 May cause an allergic skin reaction.

Table IIA 5.2-1: QRD 460: Summary of Acute Toxicity

Study	Test Substance	Result	Reference	Classification according to Dir 67/548/EEC	Classification according to Reg (EC) 1272/2008
Acute Oral	QRD 460	LD_{50} = 2000mg/kg	[redacted] J, 2011a	R22 Harmful if swallowed	Cat. 4 H302 Harmful if swallowed
Acute Dermal	QRD 460	LD_{50} > 5050mg/kg	[redacted] J, 2011b	None	None
Acute Inhalation	QRD 460	LC_{50} > 5.30 mg/L	[redacted] A, 2011	None	None
Skin Irritation	QRD 460	Not Irritant	[redacted] J, 2011c	None	None
Eye Irritation	QRD 460	Not Irritant	[redacted] J, 2011d	None	None
Skin sensitisation (LLNA)	QRD 460	Skin sensitiser	[redacted] J, 2011e	R43 May cause sensitisation by skin contact	Cat. 1 H317 May cause an allergic skin reaction
Skin sensitisation (M & K)	QRD 460	Not a sensitiser	[redacted] J, 2010	None	None
Skin sensitisation (Buehler)	QRD 420	Not a sensitiser	[redacted] J, 2009	None	None

IIA 5.2.1 Acute oral toxicity

Report:	IIA 5.2.1/01 [redacted] J (2011a). QRD 460: Acute oral toxicity study (UDP) in rats. [redacted] [redacted]. Laboratory Report No. 14695-10, 01 March 2011. Unpublished.

Guidelines

OECD 425 (2001); OPPTS 870.1100 (2002)

GLP: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

Executive Summary

The test substance, QRD 460 (100% a.i.), was evaluated for its acute oral toxicity potential in young adult female, Sprague-Dawley albino rats when administered as a gavage dose at 2000 mg/kg. Since the test substance failed the limit test, the main test was conducted following the up-and-down procedure (UDP) at 175, 550 and 2000 mg/kg. The study was terminated following the stopping rules of this procedure. The test substance was dosed at a volume ranging from 0.204 mL/kg at the 175 mg/kg level to 2.33 mL/kg at the 2000 mg/kg level. The rats were fasted overnight prior to dosing. They were assessed daily for the following 14 days for any signs of systemic toxicity and their body weights were recorded at intervals throughout the study. The animals were killed at the end of the study and were given a macroscopic examination *post mortem*.

Mortality occurred only at the 2000 mg/kg level. Clinical signs in survivors included activity decrease, body tremors, diarrhoea, piloerection, salivation and staggered gait, which were no longer evident by Day 3. Surviving animals showed weekly body weight gain during the study, except in one animal that lost weight between Days 7 and 14. Abnormal necropsy findings, pertaining to fur, lungs and contents of the stomach/intestines, were seen only in animals that died.

The acute oral LD_{50} of QRD 460 was estimated to be 2000 mg/kg in female albino rats.

Materials:

Test Material:	QRD 460
Description:	Colourless low viscosity liquid
Lot/Batch number:	AQ421-89
Purity:	100% a.i.
CAS#:	Not reported
Stability of test compound:	Not reported

Vehicle and/or positive control: None.

Test Animals:

Species	Rat
Strain	Sprague-Dawley albino
Age/weight at dosing	Young adult / 158-195 g (fasted weight)
Source	[redacted]
Housing	Individually in suspended, wire bottom, stainless steel cages
Acclimatisation period	5 days
Diet	[redacted] ad libitum, except for approximately 16 hours prior to dosing.
Water	Municipal water ad libitum
Environmental conditions	Temperature: 19-21°C Humidity: 24-89% Air changes: 10-12 per hour Photoperiod: 12 hour light, 12 dark cycle

Study Design and Methods:

In-life dates: Start: 16 December 2010 End: 24 January 2011

Animal assignment and treatment: In an acute oral toxicity study, a total of 12, young adult female Sprague-Dawley albino rats were given a single oral dose of QRD by gavage, following an overnight fast. Since the test substance failed the limit test, the main test was conducted following the up-and-down procedure (UDP) at 175, 550 and 2000 mg/kg. The study was terminated following the stopping rules of this procedure. The test substance was administered as received and was not diluted. An individual dose was calculated for each animal based on its fasted body weight and dosed at a volume ranging from 0.204 mL/kg at the 175 mg/kg level to 2.33 mL/kg at the 2000 mg/kg level.

Observations for mortality and clinical/behavioural signs of toxicity were made at least three times on the day of dosing (Day 0) and at least once daily thereafter for 14 days. Individual body weights were recorded just prior to dosing and on Days 7 and 14, or at the time of discovery after death.

On Day 14 after dosing, each surviving animal was euthanized by an overdose of CO_2. All the animals were given a gross necropsy and all abnormalities were recorded.

Statistics: The LD_{50} value with 95% confidence interval was calculated using the AOT425 Stat Program supplied by the EPA.

Results and Discussion

Mortality: Five out of six rats died at the 2000 mg/kg level. There were no mortalities at the other dose levels.

Table IIA 5.2.1-1: Acute oral toxicity of QRD 460 in the rat, application scheme and mortality data

Animal number	Dose (mg/kg bodyweight)	Dose Vol (mL	Survival
81 (limit test)	2000	0.38	Died on day 2
82	2000	0.39	Died on day 3
83	175	0.03	Survived
84	550	0.12	Survived
85	550	0.11	Survived
86	550	0.12	Survived
87	2000	0.45	Survived
88	2000	0.39	Died on day 1
89	550	0.12	Survived
90	2000	0.43	Died on Day 2
91	550	0.10	Survived
92	2000	0.46	Died on day 4

Clinical observations: Clinical signs included activity decrease, body tremors, diarrhoea, piloerection, salivation and staggered gait. Surviving animals were asymptomatic by day 3.

Body weight: Surviving animals showed weekly body weight gain during the study, except in one animal that lost weight between days 7 and 14.

Necropsy: Gross necroscopy on animals that died on test revealed wet/crushed/matted fur, discoloured lungs and contents in the gastrointestinal tract. The gross necropsy on surviving animals revealed no observable abnormalities.

Conclusion

The acute oral LD_{50} of QRD 460 was estimated to be 2000 mg/kg in female albino rats.

([redacted] J, 2011a)

IIA 5.2.2 Acute dermal toxicity

Report:	IIA 5.2.2/01; [redacted] J (2011b). QRD 460. Acute dermal toxicity study in rats. [redacted]. Laboratory Report No. 14696-10, 01 February 2010. Unpublished.

Guidelines

OECD 402 (1987): OPPTS 870.1200 (1998)

GLP: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

Executive Summary

A group of five male and five female, young adult Sprague-Dawley rats were dermally exposed to 5050 mg (6.19 mL/kg) QRD 460 (100% a.i)/kg body weight. The test substance was tested as supplied. Test sites (not less than 10% of total body surface) were covered with an occlusive dressing for approximately 24 hours, after which the dressing was removed and the skin cleansed using clean water. The animals were assessed daily for the following 14 days for any signs of systemic toxicity. Observations for evidence of dermal irritation were made at approximately 60 minutes after removal of the wrappings and on days 4, 7, 11 and 14. Body weights were recorded just prior to dosing

and on days 7 and 14. At the end of the study the animals were killed and subjected to a macroscopic examination *post mortem*.

No mortality occurred during the study. There were no clinical signs of toxicity at any time throughout the study. Signs of dermal irritation included desquamation and alopecia on Day 4. Animals exhibited weekly weight gain, with the exception of one animal that lost weight between Days 0 and 7. The gross necropsy conducted at termination of the study revealed no observable abnormalities.

The acute dermal LD_{50} of QRD 460 is greater than 5050 mg/kg in male and female rats.

Materials:

Test Material:	QRD 460
Description:	Colourless, low viscosity liquid with a 'woodsy' odour
Lot/Batch number:	AQ421-89
Purity:	100%
CAS#:	Not reported
Stability of test compound:	Not reported
Vehicle and/or positive control:	None
Test Animals:	
Species	Rat
Strain	Sprague-Dawley
Age/weight at dosing	Young adult / 253-305- g (males) and 175-209 g (females)
Source	[redacted]
Housing	Individually in suspended, wire bottom, stainless steel cages
Acclimatisation period	5 days
Diet	[redacted] ad libitum
Water	Municipal water ad libitum
Environmental conditions	Temperature: 19-21°C Humidity: 29-86% Air changes: 10-12 per hour Photoperiod: 12 hour light/ 12 dark cycle

Study Design and Methods:

In-life dates: Start: 16 December 2010 End: 30 December 2010

Animal assignment and treatment: A group of five male and five female, young adult Sprague-Dawley rats were dermally exposed to 5050 mg QRD 460/kg bodyweight. The test substance was used undiluted, as supplied. Each animal was prepared on the day prior to treatment by clipping the dorsal surface of the trunk free of hair to expose not less than 10% of the total body surface area. Care was taken to avoid abrading the skin. Only those animals with exposure areas free of pre-existing skin irritation or defects were used for this study. All animals were treated with 5050 mg/kg (6.19 mL/kg) of undiluted test substance, evenly applied in a thin, uniform layer. The area of application was covered with a 2 x 4 inch surgical gauze patch secured with non-irritating adhesive tape. The trunk of each animal was then wrapped with vet wrap which was secured in place with non-irritating adhesive tape to prevent possible ingestion of the test substance. The application period was 24 hours. After 24 hours, the wrappings were removed. The test sites were gently washed with room temperature tap water and a clean cloth to remove as much residual test substance as possible.

Observations for mortality and clinical/behavioural signs of toxicity were made at least three times on the day of dosing (Day 0) and at least once daily thereafter for 14 days. Individual body weights were recorded just prior to

dosing and on Days 7 and 14. Observations for dermal irritation were made approximately 60 minutes after removal of the wrappings, and on Days 1, 4, 7, 11 and 14. On Day 14 after dosing, each animal was euthanized by an overdose of CO_2. All study animals were subjected to gross necropsy and all abnormalities were recorded.

Statistics: The LD_{50} value was estimated (limit dose, no mortality).

Results and Discussion

Mortality: There was no mortality.

Clinical observations: All animals appeared normal for the duration of the study. Signs of dermal irritation included desquamation and alopecia on Day 4.

Body weight: Animals exhibited weekly weight gain, with the exception of 2 of 10 animals that lost weight between Days 0 and 7.

Necropsy: The gross necropsy conducted at study termination revealed no observable abnormalities.

Conclusion

The acute dermal LD_{50} of QRD 460 is greater than 5050 mg/kg in male and female rats.

(C J, 2011b)

IIA 5.2.3 Acute inhalation toxicity

Report:	IIA 5.2.3/01 A, 2011. QRD 460: Acute inhalation toxicity study in rats. Laboratory Report No. 14697-10, 21 February 2011. Unpublished.

Guidelines

OECD 403 (1981); OPPTS 870.1300 (1998)

GLP: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

Executive Summary

The test substance, QRD 460 (100% a.i.) was evaluated for its acute inhalation toxicity potential in young adult Sprague-Dawley albino rats. Five males and 5 females were exposed nose-only for 4 hours to an aerosol generated from the undiluted liquid test substance at a level of 5.30 mg/L. The concentration of the test substance in the exposure atmosphere was determined analytically once per hour and nominally at the end of the exposure. Following exposure, animals were retained for a 14 day observation period during which time they were observed at least once daily for clinical signs, and body weights were recorded just prior to the inhalation exposure, and on days 7 and 14. At the end of the study all animals subjected to gross necropsy.

No animals died during the study. The only prominent clinical signs were decreased activity and piloerection. All five males and four females lost weight during the first week, and one other female lost weight during the second week, but all females and four males had an overall weight gain during the study. The gross necropsy revealed no observable abnormalities except gas in the stomach of all animals.

The acute inhalation LC_{50} of QRD 460 is greater than 5.30 mg/L in male and female albino rats.

Materials

Test Material:	QRD 460
Description:	Colourless, low viscosity liquid with a 'woodsy' odour
Lot/Batch number:	AQ421-89
Purity:	100%
CAS#:	Not reported
Stability of test compound:	Not reported

Vehicle and/or positive control: None

Test Animals:

Species	Rat
Strain	Sprague-Dawley
Age/weight at dosing	Approximately 8 weeks old; 301-314 g (males); 186-229 g (females) at the start of exposure
Source	[redacted]
Housing	Individually in suspended, wire bottom, stainless steel cages
Acclimatisation period	5 days
Diet	[redacted] ad libitum, except during exposure
Water	Mains water *ad libitum* except during exposure
Environmental conditions	Temperature: 18-21°C Humidity: 28-89% Air changes: 10-12 air changes/hour Photoperiod: 12 hours light / 12 hours dark

Study Design and Methods:

In-life dates: Start: 21 January 2011 End: 4 February 2011

Exposure conditions: Trial assays were conducted to determine which method(s) of aerosolizing the test substance into the exposure chamber would produce an acceptable concentration and mass median aerodynamic diameter (MMAD).

Animal assignment and treatment: Five male and 5 female, young adult Sprague Dawley rats were exposed nose-only for 4 hours to an aerosol generated from QRD 460 at a level of 5.30 mg/L.

Prior to the start of the study they were examined to ensure that they were physically normal and exhibited normal activity. Observations for mortality and signs of pharmacologic and/or toxicological effects were made frequently on the day of exposure and then at least once daily thereafter for 14 days. Body weights were recorded just prior to exposure and on days 7 and 14. At the end of the scheduled period the animals were killed and examined *post mortem.*

Table IIA 5.2.3-1: Mortality / animals treated

Exposure concentration mg/L	Mortality (Number dead / total)		
	Males	Females	Combined
5.30	0/5	0/5	0/10

Generation of the test atmosphere / chamber description: A 500 L nose-only stainless steel, dynamic flow inhalation chamber was used with polycarbonate tubes which were inserted into 10 designated individual ports. The aerosol was generated by pumping the test substance into a pressure operated air atomizer, then spraying the resulting aerosol directly into the exposure chamber. Air flow into the chamber was maintained through the use of a calibrated orifice plate at a rate of 25.8 air changes per hour. Air flow was recorded at 30 minute intervals during the exposure period, and was sufficient to ensure an oxygen content of at least 19% of the exposure atmosphere. Temperature and humidity were recorded at 30 minute intervals during the exposure period from a humidity/ temperature pen inserted in an unused port of the exposure chamber.

The animals were exposed to an aerosol generated from the undiluted liquid test substance for a period of four hours. When 99% concentration (t-99) was attained, the animals that were individually housed in polycarbonate exposure tubes were inserted into a 500 L stainless steel nose-only inhalation chamber for the specified exposure period. At the termination of the exposure period, the animals were returned to their stock laboratory cages.

Test atmosphere concentration: The concentration of test substance in the exposure atmosphere (taken from the breathing zone of the animals) was determined gravimetrically once per hour and nominally at the end of the exposure. The analytical determination was made using a spectrophotometer. The nominal concentration was determined by dividing the loss in weight of the test substance after the exposure by the total volume of air that passed through the chamber.

Particle size distribution: Particle size, taken from the breathing zone of the animals, was determined twice during the exposure, using a cascade impactor, at a rate of 8.6 L/minute for a duration of 30 seconds. The MMAD and particle size distributions are calculated from these data by a computer program utilizing probit analysis.

Table IIA 5.2.3-2: Summary of acute study test atmosphere characteristics

Parameter		
Mean exposure concentration	5.30 mg/L	
Nominal concentration	45.2 mg/L	
Particle size MMAD; GSD	2.6, 3.5 µm; 4.8, 5.5 (at 1 hour and 2 hours into exposure respectively)	
Size range (µm)	% in size range	
	Run 1 (1 hour into exposure)	Run 2 (2 hours into exposure)
Particles >16.6 µm	7.02	0.00
Particles 9.9-16.6 µm	0.00	3.45
Particles 4.0-9.9 µm	7.02	5.17
Particles 2.4-4.0 µm	5.26	15.52
Particles 1.5-2.4 µm	26.32	15.52
Particles 0.8-1.5 µm	21.05	17.24
Particles 0.5-0.8 µm	21.05	17.24
Particles 0.3-0.5 µm	12.28	15.52
Particles 0.0-0.3 µm (backup filter)	0.00	10.34
Air atomizer setting: Sprayer air flow	14.2 L/min	
Air atomizer setting: Sample intake	6.0 mL/min	
Air flow rate	215 Lpm (n=9)	
Temperature	22°C (n=9)	
Humidity	32% (n=9)	

Statistics: In order to calculate a mean exposure, the Mean Value Theorem of Calculus was used to properly weight the concentration, since the concentrations could not be measured continuously. This method weights concentrations

based on the time span of each concentration. A concentration can be calculated for each minute, which better represents the exposure concentration received by each animal.

The acute inhalation LC_{50} was estimated (limit test, no mortalities).

Results and Discussion

Mortality: There were no deaths during the exposure or observation periods.

Clinical observations: Prominent in-life observations were decreased activity and piloerection. Animals were asymptomatic by day 7.

Body weight: All animals exhibited weekly weight gain during the study.

Necropsy: The gross necropsy revealed no observable abnormalities.

Conclusion

The acute inhalation LC_{50} of QRD 460 is greater than 5.30 mg/L in male and female albino rats.

([redacted] A, 2011)

IIA 5.2.4 Skin irritation

Report:	IIA 5.2.4/01 [redacted] J (2011c). QRD 460 - Acute dermal irritation study in rabbits. [redacted]. Laboratory Report No. 14699-10, 18 February 2011. Unpublished.

Guidelines

OECD 404 (2002); GPPTS 870.2500 (1998)

GLP: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

Executive Summary

In a primary dermal irritation study, three, young adult (1 male and 2 females), New Zealand White rabbits were given a single dermal application of 0.5 mL of undiluted QRD 460 (100% w/w a.i.). The test substance applied to a single intact skin site, approximately 2.5 cm x 2.5 cm on the dorsal trunk for 4 hours under a semi occlusive dressing. The application sites were observed for erythema and oedema and any other signs of skin irritation at 1, 24, 48 and 72 hours and 7, 10 and 14 days after bandage removal. Erythema and oedema were each scored on a 0-4 scale.

Very slight erythema was seen in two animals at the 24 – 72 hour readings. Oedema was not observed at any time during the study. Irritation effects persisted to day 14 of the study. One animal died on day 10 but it was not considered related to the administration of the test substance.

Under the conditions of this study, QRD 460 is considered not to require classification or labelling for skin irritancy potential.

Materials:

Test Material:	QRD 460
Description:	Colourless, low viscosity liquid with a 'woodsy' odour
Lot/Batch number:	AQ421-89

Purity:	100%
CAS#:	Not reported
Stability of test compound:	Not reported
Vehicle and/or positive control:	None
Test Animals:	
Species	Rabbit
Strain	New Zealand White
Age/weight at dosing	Approximately 14 weeks / 3.000-3.200 kg
Source	[redacted]
Housing	Individually in suspended, wire-bottom, stainless steel cages
Acclimatisation period	5 days
Diet	Lab Rabbit Diet #5321 ([redacted]) 8 oz per day
Water	Municipal water *ad libitum*
Environmental conditions	Temperature: 19-21°C Humidity: 32-93% Air changes: 10-12 air changes/hour Photoperiod: 12-hour light/dark cycle

Study Design and Methods:

In-life dates: Start: 21 December 2010 End: 04 January 2011

Animal assignment and treatment: In a primary dermal irritation study, three, young adult (1 male and 2 females), New Zealand White rabbits were given a single dermal application of 0.5 mL of QRD 460 (100% w/w a.i.).

The day before treatment, the dorsal area of the flank was clipped free of hair to expose an area at least 8 x 8 cm. Only those animals with exposure areas free of pre-existing skin irritation or defects were selected for testing. A single intact exposure site was selected as the test site while the contralateral intact site served as a control site.

On Day 0, 0.5 mL of undiluted test substance was applied to each test site and covered with a 4 ply surgical gauze patch measuring 2.5 x 2.5 cm. Each patch was secured in place with a strip of non-irritating adhesive tape. The entire trunk of each animal was loosely wrapped with a semi-permeable dressing (orthopaedic stockinette) and secured on both edges with strips of tape to retard evaporation of volatile substances and to prevent possible ingestion of the test substance.

After four hours, the patches and wrappings were removed. The test sites were gently washed with room temperature tap water and a clean cloth to remove as much residual test substance as possible.

The animals were checked daily for signs of systemic toxicity and mortality. The test sites were observed for erythema and oedema formation, and any other dermal defects or irritation, at 1, 24, 48 and 72 hours and 7, 10 and 14 days after unwrap. Erythema and oedema were each scored on a 0-4 scale. For each animal, all of the erythema and oedema scores through 72 hours were added, and the sum was divided by 4 to obtain an individual irritation score. The primary irritation index was determined by calculating the mean of the irritation scores for all the animals and was used to obtain a rating for the test substance.

Results and Discussion

One animal died on day 10. Death was not considered related to the administration of the test substance.

Very slight erythema was present at the 24 hour – day 14 observations. Oedema was not observed at any time throughout the study. Other signs of irritation included desquamation.

Table IIA 5.2.4-1: Individual and mean skin irritation scores of QRD 460

Time	Erythema			Oedema		
Animal number	5454	5453	5455	5454	5453	5455
after 1 hour	0	0	0	0	0	0
after 24 hours	0	1	1	0	0	0
after 48 hours	0	1	1	0	0	0
after 72 hours	0	1	1	0	0	0
mean score 24-72 h	0	1	1	0	0	0
after 7 days	0	1	1	0	0	0
after 10 days	0	1	1	0	0	0
after 14 days	-	1	0	-	0	0

The Primary Irritation Index (PII) of 0.3 out of a possible 8.0 was obtained from the 1, 24, 48 and 72 hour observations.

Conclusion

Under the conditions of this study, QRD 460 is considered not to require classification or labelling for skin irritancy potential.

([redacted] J, 2011c)

IIA 5.2.5 Eye irritation

Report: IIA 5.2.5/01 [redacted] J (2011d). QRD 460: Acute eye irritation study in rabbits. [redacted]. Laboratory Report No. 14698-10, 01 February 2011. Unpublished.

Guidelines

OECD 405 (2002); OPPTS 870.2400 (1998)

GLP: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

Executive Summary

In a primary eye irritation study, 0.1 mL by volume of QRD 460 (100% a.i.) was placed into the conjunctival sac of the right eye of each of a group of 3 New Zealand White rabbits (2 males and 1 female). The grades of ocular reaction were recorded at 1, 24, 48 and 72 hours, and at 4 and 7 days after treatment. The corneas of all treated eyes were examined immediately after the 24 hour observation with a fluorescein sodium ophthalmic solution. All treated eyes were washed with room temperature deionised water for one minute immediately after recording the 24-hour observation. Corneal opacity, iritis and conjunctival redness, chemosis and discharge were scored based on the Draize numerical scale.

Slight conjunctival redness was present in one animal at the 1 and 24 hour readings. Slight chemosis was present in one animal at the 1 hour reading but had gone by the 24 hour reading. Slight or moderate conjunctival discharge was present in one animal at the 1 and 24 hour readings, and in another animal at 24 hours but had cleared by the 48 hour readings.

No abnormal findings were observed in the treated eye of any animal 7 days after treatment.

Under the conditions of this study, QRD 460 is considered not to require classification or labelling for ocular irritation potential.

Materials:

Test Material:	QRD 460
Description:	Colourless, low viscosity liquid with a 'woodsy' odour
Lot/Batch number:	AQ421-89
Purity:	100%
CAS#:	Not reported
Stability of test compound:	Not reported
Vehicle and/or positive control:	None
Test Animals:	
Species	Rabbit
Strain	New Zealand White
Age/weight at dosing	Approximately 13 weeks / 2.725-3.050 kg
Source	[redacted]
Housing	Individually in suspended, wire-bottom, stainless steel cages
Acclimatisation period	5 days
Diet	Lab Rabbit Diet #5321 ([redacted]) approximately 8 oz per day
Water	Municipal water ad libitum
Environmental conditions	Temperature: 20-21°C Humidity: 37-92% Air changes: 10-12 air changes/hour Photoperiod: 12-hour light/dark cycle

Study Design and Methods:

In-life dates: Start: 20 December 2010 End: 23 December 2010

Animal assignment and treatment: In a primary eye irritation study, QRD 460 (100% a.i.) was assessed for eye irritation in a group of 3 New Zealand White rabbits (2 males and 1 female). Only animals without eye defects or irritation were selected for testing.

On Day 0, a dose of 0.1 mL of the undiluted test substance was placed into the conjunctival sac of the right eye of each animal by gently pulling the lower lid away from the eyeball to form a cup into which the test substance was dropped. The lids were gently held together for one second to prevent loss of material. The untreated left eyes served as comparative controls.

The treated eyes of all animals were examined without magnification under white room lighting, and (if needed), an additional source of white light or a handheld flashlight. The grades of ocular reaction recorded at 1, 24, 48 and 72 hours, and at 4 and 7 days after treatment. The corneas of all treated eyes were examined immediately after the 24 hour observation with a fluorescein sodium ophthalmic solution. An ocular transilluminator was used to enhance visualization of fluorescein staining. Any of the corneas which exhibited fluorescein staining at the 24 hour

observation were re-examined with the fluorescein sodium ophthalmic solution at each consecutive observation until fluorescein staining of the cornea no longer occurred. All treated eyes were washed with room temperature deionized water for one minute immediately after recording the 24 hour observation.

Individual irritation scores for each animal at each scheduled observation were determined using a numerical grading scale similar to the Draize scale. An average irritation score for each scheduled observation for all eyes was then determined, based on the number of animals tested. A maximum average irritation score was derived from the observation yielding the highest average irritation score. The maximum average irritation score was used to rate the test substance. Any corneal involvement or iridic irritation with a score of 1 or more is considered positive. Any conjunctival irritation (redness or chemosis) with a score of 2 or more is considered positive.

Results and Discussion

Slight conjunctival redness was present in one animal at the 1 and 24 hour readings. Slight chemosis was present in one animal at the 1 hour reading but had gone by the 24 hour reading. Slight or moderate conjunctival discharge was present in one animal at the 1 and 24 hour readings, and in another animal at 24 hours but had cleared by the 48 hour readings.

No abnormal findings were observed in the treated eye of any animal 7 days after treatment.

The maximum average irritation score of 2.0, obtained 1 and 24 hours after treatment, was used to rate QRD 460 minimally irritating. Fluorescein staining did not occur in any of the eyes.

Table IIA 5.2.5-1: Eye irritation scores of QRD 460

Time	Cornea			Iris			Conjunctiva					
							Redness			Chemosis		
Animal number	5444	5446	5445	5444	5446	5445	5444	5446	5445	5444	5446	5445
after 1 hour	0	0	0	0	0	0	1 d	0	0	1	0	0
after 24 hours	0	0	0	0	0	0	1 d	0 d	0	0	0	0
after 48 hours	0	0	0	0	0	0	0	0	0	0	0	0
after 72 hours	0	0	0	0	0	0	0	0	0	0	0	0
mean scores 24-72h	0	0	0	0	0	0	0.3	0	0	0	0	0
after 4 days	0	0	0	0	0	0	0	0	0	0	0	0
after 7 days	0	0	0	0	0	0	0	0	0	0	0	0

d - discharge

Conclusion

Under the conditions of this study, QRD 460 is considered not to require classification or labelling for ocular irritation potential.

([redacted] J, 2011d)

IIA 5.2.6 Skin sensitisation

Report:	IIA 5.2.6/01 [redacted] J (2011e) QRD 460 Skin sensitisation Local Lymph Node Assay in Mice. [redacted]. Laboratory Report No. 14700-10, 01 February 2011. Unpublished.

Guidelines

OPPTS 870.2600 (2003): OECD 429 (2010)

GLP: Signed and dated GLP and Quality Assurance statements were provided.

Executive Summary

A skin sensitisation study was conducted on mice to determine if test substance QRD 460 possesses a significant potential to cause skin sensitisation. Five females were assigned to each of three groups. The test groups were treated with the appropriate dilution (25% or 50%) in acetone/olive oil, or undiluted test substance. Each animal received 25 µL to the dorsum of each ear. The animals were treated once daily for three days. After a two-day rest period, all animals were injected with tritrated methyl-thymidine in the tail vein. Five hours later, the animals were sacrificed, and the draining auricular lymph nodes removed and prepared for cell suspension and scintillation counting. A vehicle control group of five females was run concurrently, treated in the same manner. A positive control group of five females was also run concurrently, treated with 85% alpha-hexylcinnamaldehyde in acetone:olive oil.

The test substance produced a stimulation index of ≥ 3 in all groups of test animals, and is therefore considered a sensitiser (defined as producing a positive response).

Materials

Test Material:	QRD 460
Description:	Colourless, low viscosity liquid with 'woodsy' odour
Lot/Batch #:	AQ421-89
Purity:	100%
Stability of test compound:	Not reported

Vehicle and/or positive control: Acetone:olive oil / alpha-hexylcinnamaldehyde

Test Animals:	
Species	Mouse
Strain	CBA/J
Age/weight at dosing	Young adults / 17.5 – 23.2 g
Source	[redacted]
Housing	Individually.
Acclimatisation period	At least 5 days
Diet	[redacted] *ad libitum*
Water	Municipal water supplied by an automatic system *ad libitum*
Environmental conditions	Temperature: 19-23°C Humidity: 25-69% Air changes: 10-12 changes/hour Photoperiod: Artificial, 12 hours light / 12 hours dark.

Study Design and Methods

In-life dates: Start: 15 December 2010 End: 20 December 2010

Five females were selected for each of three test groups. On days 1, 2 and 3 each test animal received an open application of 25 µL of an appropriate dilution (25% or 50%) of the test substance, or 100% test substance undiluted, to the dorsum of both ears. The vehicle control group (5 females) was treated in the same way as test animals, but with vehicle alone (acetone:olive oil) instead of test substance. The positive control group was treated with 85% alpha-hexylcinnamaldehyde in acetone:olive oil. All test and control animals were rested on days 4 and 5.

On day 6, all test and control animals were injected in the tail vein with 250 µL of 0.01 M phosphate-buffered saline (PBS) containing 20 µCi of [methyl,1^1,2^1-^{3}H] Thymidine. Five hours later, the animals were sacrificed, the draining auricular nodes excised and pairs from each individual processed.

A single cell suspension was prepared by gentle disintegration through 200 mesh stainless steel gauze. The cells were washed twice with an excess of PBS and precipitated with 5% trichloroacetic acid (TCA) at 4ºC for 18 hours. The pellets were resuspended in 1 mL of TCA and transferred to 10 mL of scintillation fluid. Incorporation of tritrated

thymidine was measured by liquid scintillation counting as disintegrations per minute (DPM) from the paired lymph nodes of each animal, and mean DPM/animal was calculated for each group.

Results and Discussion

One test group II animal and two test group III animals lost weight during the study. All test animals appeared normal for the duration of the study.

Individual DPM counts are presented in Table IIA 5.2.6-1. The Stimulation Index (SI) or test/vehicle control ratio derived for each test group based on the group mean DPM was as follows:

Table IIA 5.2.6-1: Radiolabel incorporation into lymph-nodes of mice treated with QRD 460

Animal Group	Test Substance Concentration	Average Count per Mouse	No. of Mice in Group	Test/Vehicle Control Ratio
Vehicle Control	NA	485	5	NA
Test Group I	25%	2182	5	4.5
Test Group II	50%	1650	5	3.4
Test Group III	100%	3017	5	6.2
Positive Control	NA	7378	4#	15.2##

NA – Not Applicable
- One animal found dead on Day 4
- Positive control used validate procedures

Conclusions

QRD 460 produced a stimulation index of ≥ 3 in all groups of test animal, and is therefore considered a sensitiser (defined as producing a positive response).

([redacted] J, 2011e)

Report: IIA 5.2.6/02 [redacted] J (2010). QRD 460 Guinea pig maximisation test for topically applied test substance. [redacted].
Laboratory Report No. 14163-10, 19 October 2010. Unpublished.

Guidelines:

OECD 406 (1992); OPPTS 870.2600 (2003)

GLP: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

Executive Summary

The sensitisation potential of QRD 460 was assessed using a method based on the maximisation test of Magnusson and Kligman (1969).

Groups of 10 per sex (test group) and 5 per sex (control group), young adult albino Hartley guinea pigs were used for the main study. Two main procedures were involved; (a) the induction of an immune response; (b) a challenge of that response.

For the main study, the concentrations used were 5% (v/v) in cottonseed oil for the induction intradermal injections, 100% for the topical induction applications and 100% for the challenge applications.

A positive control study was conducted using essentially the same methodology and using 85% alpha-hexylcinnamaldehyde as the test substance. The method used an intradermal induction of a 5% v/v preparation in corn oil with and without adjuvant (50:50 v/v) for intradermal injections; 100% for the topical induction and challenge phase.

Following challenge with the undiluted test sample, no erythema was seen in test or control animals. The net response was 0%.

No erythematous reactions were seen in any animal, test or control, following challenge with vehicle alone. The net response was zero.

In the positive control study, all animals of the test group exhibited discrete to moderate erythema after the challenge treatment, and none of the control group exhibited erythema. Alpha-hexylcinnamaldehyde was considered to elicit an extreme sensitisation response in previously induced guinea pigs thereby confirming the sensitivity of the test.

Since 0% of the test group animals exhibited scores greater than zero, QRD 460 was given a sensitisation potency rating of non-sensitizer.

Materials:

Test Material:	QRD 460
Description:	Colourless/slightly yellow liquid with a 'woodsy' odour
Lot/Batch number:	408-5-1
Purity:	100% a.i.
CAS#:	Not reported
Stability of test compound:	Not reported
Vehicle and/or positive control:	vehicle – cottonseed oil; positive control – Alpha-Hexylcinnamaldehyde, tech, 85% (vehicle: corn oil)

Test Animals:

Species	Rat
Strain	Sprague-Dawley
Age/weight at dosing	Approximately 8 weeks old; 298-315 g (males); 193-227 g (females) at the start of exposure
Source	[redacted]
Housing	Individually in suspended, wire bottom, stainless steel cages
Acclimatisation period	5 days
Diet	[redacted] ad libitum, except during exposure
Water	Mains water ad libitum except during exposure.
Environmental conditions	Temperature: 20-22°C Humidity: 55-93% Air changes: 10-12 air changes/hour Photoperiod: 12 hours light / 12 hours dark

Study Design and Methods:

In-life dates:	Start: 25 August 2010	End: 18 September 2010 (main study)
	Start: 10 March 2010	End: 3 April 2010 (positive control study)

Animal assignment and treatment: The sensitisation potential of QRD 460 was assessed using a method based on the maximisation test of Magnusson and Kligman (1969).

Groups of 10 per sex (test group) and 5 per sex (control group), young adult albino Hartley guinea pigs were used for the main study. Two main procedures were involved; (a) the induction of an immune response; (b) a challenge of that response.

Induction: The animals were treated on day 0 by making three pairs of symmetrical intradermal injections on the upper back of each animal within a 4 x 6 cm exposure area running laterally across the shoulders. For the test group animals, the first pair of injections (one on each side of the spinal column and approximately 3.5 cm apart), consisting of Freund's Complete Adjuvant diluted to 50% v/v in saline, was made at the anterior edge of the exposure area. The second pair of injections, consisting of 5% v/v test substance in cottonseed oil, was made approximately 0.5 cm behind the first pair. The third pair of injections, consisting of a 50:50 mixture of Freund's Complete Adjuvant (diluted to 50% v/v in saline) and a solution of 5% v/v test substance in cottonseed oil was made approximately 0.5 cm behind the second pair of injections. Control group animals received the same injections with the vehicle substituted for the test substance in the second and third pairs of injections. All injections were within a 2 x 4 cm area of the 4 x 6 cm exposure area. A volume of 0.1 mL was administered at each site.

On Day 7, 0.5 mL of undiluted test substance was applied to the exposure area of each test animal to cover the intradermal injection sites. A 25 mm glass fibre filter patch was used to cover the dose site. The patch was then occluded with an adhesive masking tape and secured in place with an elastic adhesive wrap wound around the torso of the animal. Control animals received 0.5 mL of cottonseed oil and a 25 mm glass fibre filter patch was placed over the dose sites. The wrappings and patches were removed after 48 hours. Test sites 3 and 4 were observed for dermal irritation on day 10.

Challenge: On day 20, a 5 x 5 cm area was clipped on both the left and right flanks of each test and control animal. For the challenge treatment (day 21), 0.5 mL of undiluted test substance was applied topically to the right flank of each animal in a manner identical to the day 7 treatment. A 25 mm glass fibre filter patch was used to cover the dose site. A dose of 0.5 mL of cottonseed oil was applied topically to the left flank of each animal. A 25 mm glass fibre filter patch was placed on the dose site. Patches were secured as for topical induction.

Positive Controls: A positive control study was conducted using essentially the same methodology and using 85% alpha-hexylcinnamaldehyde as the test substance. The method used an intradermal induction of a 5% v/v preparation in corn oil with and without adjuvant (50:50 v/v) for intradermal injections; 100% undiluted test substance for the topical induction and challenge phase.

Results and Discussion

Mortality / Clinical observations: On days 8-9, two control animals were discovered to have prolapsed intestines and were humanely killed; one other control animal and two test animals were found dead. These deaths are considered not to be related to test substance administration.

Induction reactions and duration: Not reported.

Body weights: There were no treatment-related effects on body weight during the study.

Challenge reactions and duration: The challenge treatments with either vehicle alone or test substance alone produced no erythema in any test animals or in any control animals.

Positive control: All animals of the test group exhibited discrete to moderate erythema after the challenge treatment, and none of the animals of the control group exhibited erythema after the challenge treatment, therefore the test substance is considered an extreme sensitizer and confirmed the sensitivity of guinea pigs to the positive control material.

Table IIA 5.2.6-2: Maximisation test: Number of animals with signs of allergic skin reactions

Scored after:	24 hours	48 hours
Test group (undiluted QRD 460)	0/18	0/18
Vehicle control group (cottonseed oil)	0/7	0/6
Positive control (85% alpha-hexylcinnamaldehyde)	9/9 (0/9 control)	1/9 (0/9 control)

Conclusion

Since 0% of the test group animals exhibited scores greater than zero, QRD 460 was given a sensitisation potency rating of non-sensitizer.

Reference: Magnusson B and Kligman AM (1969). J. Invest. Dermat 52: 268-276.

(J, 2010)

Report: IIIA 7.1.6/03 J, (2009). QRD 420: Skin sensitization study in guinea pigs. . Laboratory Report No. 12572-08. Issue date 15 April 2009. Unpublished.

Guidelines

OECD 406 (1992): OPPTS 870.2600 (1998)

GLP: Signed and dated GLP and Quality Assurance statements were provided.

Executive Summary

A skin sensitization study, based on the method described by Ritz and Buehler, 1980, was conducted on 15 male and 15 female short-haired Hartley-Albino guinea pigs to determine if test substance QRD 420 produced a sensitising reaction. Animals were assigned to each of two groups, designated Groups I and II. Group I animals (5 per sex) remained untreated during the induction phase of the study and served as a naive control group. Group II animals (10 per sex), the test group, were treated with 0.4 mL of undiluted test substance (selected from previous screening). The animals were treated once weekly for three weeks, i.e. a total of three treatments. After a two-week rest period, all animals (Groups I and II) were challenged at a virgin test site with an application of 0.4 mL of undiluted test substance.

The sensitivity of guinea pigs to a positive control material, 85% alpha-hexylcinnamaldehyde, was confirmed.

QRD 420 produced no irritation in the test animals (Group II) or the naive control animals (Group I) after the challenge treatment, and therefore did not elicit a sensitizing reaction in guinea pigs.

QRD 420 was not a skin sensitiser under the conditions of the test.

Materials:

Test Material:	QRD 420
Description:	Pale yellow liquid
Lot/Batch number:	T-Y01
Purity:	67.18% technical grade a.i.
CAS#:	Not reported
Stability of test compound:	Not reported

Vehicle and/or positive control: None / positive control was 85% alpha-hexylcinnamaldehyde.

Test Animals:	
Species	Guinea pig
Strain	Hartley-Albino
Age/weight at dosing	5-6 weeks / 319-390 g males and 308-363 g females
Source	[redacted]
Housing	1-4 per cage (sexes separately) in suspended, wire bottom, stainless steel cages
Acclimatisation period	5 days
Diet	Guinea Pig Diet 5025 ([redacted]) ad libitum
Water	Municipal water ad libitum
Environmental conditions	Temperature: 15-25°C Humidity: 24-98% Air changes: 10-12 per hour Photoperiod: 12-hour light/dark cycle

Study Design and Methods:

In-life dates: Start: 4 March 2009 End: 1 April 2009 (main study)

Start: 5 June 2008 End: 5 July 2008 (positive control study)

Animal assignment and treatment: The sensitisation potential of the test substance was assessed using a method based on that described by Ritz and Buehler. Two main procedures were involved: (a) the potential induction of an immune response; (b) a challenge of that response. Young adult, Hartley albino guinea pigs were assigned to each of two groups, designated Groups I and II. Group I animals (5 per sex) remained untreated during the induction phase of the study and served as a naive control group. Group II animals (10 per sex), the test group, were treated with 0.4 mL of undiluted test substance. A preliminary irritation test was carried out to determine the highest non-irritating concentration (HNIC) of the test substance prior to the challenge dose. The HNIC selected for the challenge phase was 100%.

On the day prior to each treatment, the animals were prepared by clipping the back of the trunk free of hair to expose a longitudinal area at least 8 x 10 cm on each animal. Individual body weights were recorded on Days 0 and 31.

Induction: For each induction treatment, Group II animals were treated with 0.4 mL undiluted test substance beneath a 4 ply, 2.5 x 2.5 cm surgical gauze patch on the left front quadrant of the exposure and secured with a strip of non-irritating adhesive tape. A strip of clear polyethylene film was placed over the patch and securely taped. Each animal was then placed in a restrainer for approximately six hours. At the end of the exposure period, the animals were removed from the restrainers, the wrappings and patches were removed, and the animals were returned to their cages. Group II animals were treated once weekly for three weeks, on days 1, 8 and 15. The same treatment regimen and test site location was used for all three induction treatments. Group I animals remained untreated during the induction phase of the study.

Observations for skin reactions at each test site were made approximately 24 hours after each treatment and approximately 48 hours after the first induction treatment. Erythema was scored on a 0-3 scale.

Challenge: After a two week rest period, all animals (Groups I and II) were each challenged at a virgin test site with an application of 0.4 mL of undiluted QRD 452. The challenge treatment was on Day 29. The dose was applied in a manner identical to the induction treatments, except the test site was placed laterally on the right rear quadrant of the exposure area.

Observations for skin reactions at each test site were made approximately 24 and 48 hours after challenge. Erythema was scored on a 0-3 scale.

An average score for each time period was obtained by adding all of the scores for each time period and dividing by the number of test sites scored for that time period. The test substance is considered a sensitizer if the mean irritation scores, the total number of animals with scores, and/or the total number of scores for the virgin test site in the test group after the challenge treatment are appreciably greater than those for the naive challenge group.

Positive Controls: The sensitivity of guinea pigs to a positive control material (alpha-hexylcinnamaldehyde, 85%) was confirmed in this laboratory. Induction and challenge applications used the neat test substance.

Results and Discussion

Mortality / Clinical observations: All animals survived till the end of the study. No abnormal behaviour or clinical signs were detected.

Body weights: There were no treatment-related effects on body weight during the study.

Induction reactions and duration: There were no signs of irritation.

Challenge reactions and duration: There were no signs of irritation.

Positive control: Faint to strong erythema was seen in 10/10 animals twenty-four hours after the end of the challenge exposure and very faint to faint erythema was present in six animals at the 48 hour reading. A mean score of 1.2 for the test group after challenge treatment, when compared with the naive control group mean score of 0.1, confirmed the sensitivity of the strain of animals used and the reliability of the experimental technique.

Table IIA 5.2.6-3: Buehler test: Number of animals with positive signs of allergic skin reactions following challenge

	Test flank	
	Challenge at 100%	
Scored after:	**24 hours**	**48 hours**
Main test – test group	0/20	0/20
Main test – negative vehicle control	0/10	0/10
	Challenge at 100%	
	24 hours	**48 hours**
Positive control – test group	10/10	6/10
Positive control – vehicle control	3/10	0/10

Conclusion

QRD 420 was not a skin sensitiser under the conditions of the test.

Reference

H.L. Ritz and E.V. Buehler, "Planning, Conduct and Interpretation of Guinea Pig Sensitization Patch Tests," Current Concepts in Cutaneous Toxicity, p. 25-42, Academic Press, NY, 1980)

([redacted] J, 2009)

IIA 5.2.7 Potential/interactions of multiple active substances or products

This is not an EC data requirement

IIA 5.3 Short term toxicity

It is considered that sufficient data and information are available from other sources, as detailed below, to address the short term toxicity of QRD 460 and hence there is no need to conduct further studies. The waiver justifications below support the contention that the use of products containing QRD 460 will not result in repeated human exposure to QRD 460 by the oral, inhalation or dermal routes.

A. Oral toxicity

Proposed Uses for PPPs containing QRD 460. The representative formulation, QRD 452, contains 16.75% QRD 460 as the active ingredient. The intended uses of QRD 452 in Europe are commercial agricultural applications on plants intended for human consumption. QRD 452 has a US EPA exemption from the requirement for a tolerance (40 CFR 180.1296) based on absence of detectable residue and resultant lack of oral exposure to all populations. More importantly, for reasons outlined below, the intended use of the product is not likely to result in repeated human exposure by the oral route.

No Adverse Effects Associated with Natural Occurrence and Permitted Uses. The inherent exposure of humans to the active ingredient components through their natural occurrence or via their permitted uses as direct food additives, flavouring agents, and fragrances in food, cosmetics, and other consumer goods, including other pesticides, has not resulted in widespread reports in the published literature of adverse effects.

The components of QRD 460 (α-terpinene, p-cymene, and d-limonene) are naturally occurring in a multitude of fruits, vegetables, herbs, spices, and other foods and beverages, including coffee, tea, alcoholic beverages, baked and fried potatoes, bread and cheese. In addition to the natural occurrence, the active ingredient components of QRD 460 are permitted for use as food additives in the US and Europe, and as fragrance additives in cosmetics[18]. Although the levels are relatively low, the general public is exposed to these components through ingestion, dermal contact, and inhalation on a daily basis. According to a 2005 World Health Organization (WHO) report[19] on food additives, the per capita daily consumption of the three main components as food additives in the US and Europe, respectively, are as follows: d-limonene, 12.76 mg and 39.307 mg; p-cymene, 0.472 mg and 1.085 mg; α-terpinene, 0.093 mg and 0.032 mg.

The Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC) report Maximised Survey-derived Daily Intakes (MSDI).

$$\text{MSDI } (\mu g/\text{capita/day}) = \frac{\text{Annual production (kg)} \times 10^9\ (\mu g/kg)}{\text{Consumers} \times \text{survey response rate} \times 365\ (\text{days})}$$

Notes:
Annual production volume in one year in Europe
Consumers: estimated to be 10% of the total European population (=32,000,000)
Survey response rate: correction made to take account that data provided by industry may be incomplete (= 0.6 in Europe)

MSDI values for p-cymene of 926 µg/capita/day[20], α-terpinene of 27 µg/capita/day[21] and d-limonene of 33542 µg/capita/day[21] are reported. All were considered of no safety concern at the estimated levels of intake.

α-Terpinene is a monoterpene found in the essential oils of a variety of useful plants, including citrus, peppermint, thyme, basil, and papaya[22]. In addition, α-terpinene is listed in 21 CFR 172.515 as a food additive permitted for direct addition to food for human consumption[23].

p-Cymene occurs naturally in more than 200 foods. Oral intake of p-cymene occurs predominantly from consumption of foods such as butter, carrots, nutmeg, orange juice, oregano, raspberries, and lemon oil, and

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[18] Scientific Committee on Cosmetic Products and non-Food Products intended for consumers. (2000)The 1st update of the inventory of ingredients employed in cosmetic products. Section II. Perfume and aromatic raw materials. SCCNFP/0389/00 Final

[19] WHO (2005), Evaluation of Certain Food Additives. WHO Technical Report Series No. 928. 63rd Report of the Joint FAO/WHO Expert Committee on Food Additives.

[20] Opinion of the Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC) related to Flavouring Group Evaluation 18 (FGE.18): Aliphatic, alicyclic and aromatic saturated and unsaturated tertiary alcohols, aromatic tertiary alcohols and their esters from chemical group 6 (2006). The EFSA Journal 331, 1-77.

[21] Flavouring Group Evaluation 25, (FGE.25)[1] - Aliphatic and aromatic hydrocarbons from chemical group 31 - Scientific Opinion of the Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (2008). The EFSA Journal 918, 1-109.

[22] Cornell University, Medicinal Plants website: Medicinal Plants for Livestock, Beneficial or Toxic? [http://www.ansci.cornell.edu/plants/medicinal/plants.html]

[23] Code of Federal Regulations. Title 21, Volume 3. April 1, 2005. [URL: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=172.515

spices[24,25]. p-Cymene is currently permitted by the U.S. Food and Drug Administration (FDA) for direct addition to food for human consumption as a flavouring substance and is considered by the Flavour and Extract Manufacturers' Association (FEMA) Expert Panel to be "generally recognized as safe" (GRAS) for use as a flavouring substance.

d-Limonene is a naturally occurring, major component of lemon oil, orange oil and grapefruit oil, and is a naturally occurring, minor component in other fruits, vegetables, meats, and spices[26]. d-Limonene is widely used as a flavour and fragrance and is listed as generally recognized as safe (GRAS) in by the FDA as a food additive of flavouring, and as a fragrance additive. In addition, limonene is a registered active ingredient in 15 pesticide products used as insecticides, insect repellents, and dog and cat repellents[27] More recently, EPA has registered limonene as an herbicide. Limonene is listed in 40 CFR 189.910 as exempt from the requirement of a tolerance in pesticide formulations when applied as an inert ingredient to growing crops or to raw agricultural commodities. Health Canada's Pest Management Regulatory Agency (PMRA) has also proposed full registration for the use of d-limonene to control insects. They considered it to be of low toxicity to humans[28]

Based on the above information, it is reasonable to conclude that the general population is frequently and widely exposed to α-terpinene, p-cymene and d-limonene, through the ingestion of food products and dermal contact with plants, vegetation, and cosmetics that naturally or artificially contain these components. In addition, based on the reasons provided above, it reasonable to conclude that little, if any, of this daily exposure of the general population will be as a result of the use of the formulated product for its intended applications, food-use or otherwise.

Label Uses Minimize Exposure. The proposed label minimizes the potential for human exposure, including the potential for repeated oral exposure. QRD 452 is neither labelled for, nor to be sold or marketed for use in homes, home gardens or other residential situations, which eliminates potential oral exposure of children and toddlers. Furthermore, the maximum proposed application rate of 1.523 kg as/ha will always be applied as a dilute solution in water, therefore, applicators will never be exposed to full strength product.

Protective Clothing and Use Instructions Label instructions specify that when using the product handlers and applicators are to wear specified protective clothing. Furthermore, they are instructed to "Wash thoroughly with soap and water after handling and before eating, drinking, chewing gum or using tobacco." QRD 452 is labelled for use in commercial agricultural situations and we believe it is a reasonable expectation that the label instructions will be followed as indicated. It is reasonable to conclude that the precautions specified on the label (use of the required PPE and following the hygiene instructions) combined with the intended agricultural application sites and low application rate essentially eliminates the potential for any significant repeated oral exposure by mixers/handlers and applicators. In addition, the rapid disappearance of foliar residues (described below) would be expected to eliminate the potential for any inadvertent oral ingestion (e.g., via unwashed hand to mouth).

Rapid Foliar Residue Decline Residues of α-terpinene, p-cymene and d-limonene declined to non-detectable levels within 10 minutes after foliar applications of QRD 400 (Facin 25EC) on Primrose at an application rate of 9.35 L as/ha. Thus, the potential for any post-application oral exposure by applicators or others is essentially non-existent. AQ conducted a residue decline study titled Persistence of Facin 25% EC on Primrose (*Primula acaulis*) (MRID 47209101, see Document MII Section 4 for full details), which showed rapid dissipation of the marker components, thus supporting the argument that exposure to residual product is also of minimal concern. The study clearly shows that even at an exaggerated application rate, the three marker components are not detectable within 10 minutes of product application. Essentially, by the time the leaves have dried there is no detectable residual product. This limits the window of opportunity for hand-to-mouth exposure from foliage after application.

Results of the Primrose study are consistent with and supported by the residue decline study Agraquest Inc. conducted with QRD 400 (Facin 25EC) on tomatoes (MRID 46858903, see Document MII Section 4 for details). The tomato

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[24] The Flavor and Fragrance High Production Volume Consortia. The Terpene Consortium: Test Plan for Aromatic Terpene Hydrocarbons. p-cymene. 2002.

[25] Purdue University CROP™ Crop Resource Online Program, Mandarin Orange, Citrus reticulata.[URL: http://www.hort.purdue.edu/newcrop/morton/mandarin_orange.html]

[26] U.S. EPA, D-Limonene; Reregistration Eligibility Decision for Low Risk Pesticide; Notice of Availability, FR 70 (12): 3022-3024 (January 19, 2005)

[27] U.S. EPA Reregistration Eligibility Decision (RED) – Limonene. September 1994

[28] Health Canada. Proposed Registration Decision PRD2010-21, d-Limonene. 27 Aug 2010. http://www.hc-sc.gc.ca/cps-spc/pest/part/consultations/_prd2010-21/d-limonene-eng.php

study demonstrated rapid dissipation of the active constituents of the extract of *C. ambrosioides*, at 0, 3, 6, 12, and 24 hr post-treatment. All constituents were less than the limit of quantitation (<LOQ) of 0.01 mg/kg at all-time intervals except for d-limonene, which was attributed to background d-limonene naturally occurring in tomatoes. The product was applied four times using an application rate of 2.010 kg as/ha.

Finally, results of a residue decline study performed for AgraQuest Inc. with the QRD 452 product (then called QRD 416) showed that residues of the three major terpene components declined to non-detectable levels within one hour after foliar application on mustard greens at application rates of 2690 – 2914 g as/ha. In the study, QRD 400/QRD 416: Residue Levels of Terpenes in Mustard Greens from a Trial Conducted in California during 2007 (Study ID No. 77SRU07R-1, MRID 47548301, see Document MII Section 4 for full details), the product was applied three times at specified intervals and the plants were sampled at 0, 1, and 4 hours after the third application.

In summary, results of all three residue decline studies demonstrate that multiple applications of QRD 400 or QRD 452 result in no detection of residue shortly after application and no accumulation of residues over multiple applications. Not only does this limit the opportunity for accidental hand-to-mouth exposure from foliage after application, the rapid dissipation of the terpene components and absence of residues should ensure minimal concern over consumption of these substances through crops treated with the product.

Lack of Acute Oral Toxicity Studies on QRD 460 and QRD 452 establish that the active substance is not highly acutely toxic to mammals. Rat LD_{50s} were 2,000 mg/kg and >5,000 mg/kg in studies with the active substance and formulated EC products, respectively.

Literature Sources: Of the components in QRD 460, most published data are available for d-limonene**.** The International Programme on Chemical Safety[29] reviewed d-limonene in 1998 and considered it to "essentially non-toxic". Following oral or intraperitoneal administration, the target organ in animals (except male rats) was the liver. Exposure affects the activity of different liver enzymes, liver weight, cholesterol levels and bile flow; changes have been reported in mice, rats, and dogs (Maltzman *et al*., 1991[30], Kanerva *et al*., 1987). Whilst in male rats, d-limonene exposure causes damage to the kidneys, the male rat specific protein, alpha2u-globulin, is considered to play a role in the development of kidney lesions, and therefore these effects are considered not to be relevant for human risk assessment.

Ariyoshi *et al*., (1975)[31] reported rats receiving an oral dose of 400 mg/kg bw for 30 days showed a 20-30% increase in the amount and activity of liver enzymes (cytochrome P-450, cytochrome b5, aminopyrine demethylase, and aniline hydroxylase), increased relative liver weight, and decreased cholesterol levels. No histopathological changes were seen in the liver.

d-Limonene (0, 2, 5, 10, 30, and 75 mg/kg bw/day) was administered orally to male rats, 5 days/week for 13 weeks (Webb et al., 1989[32]). The no-observed-effect level (NOEL), based upon histological examination of the kidneys, was considered to be 5 mg/kg bw per day (but as stated previously, these effects are considered male rat specific and not to be relevant for human risk assessment). No histopathological effects were seen in the liver. The LOEL for increased liver weight was 75 mg/kg bw/day, the highest dose tested and the no-observed-adverse-effect level (NOAEL) for effects in the liver was 30 mg/kg bw/day. The amount and activity of different liver enzymes were not investigated; therefore the increased relative liver weight may have been due to enzyme induction.

[29] IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene

[30] Maltzman TH (1991) Anticarcinogenic mechanism of action of dietary limonene during the initiation stage of DMBA-induced mammary carcinogenesis. Dissertation abstracts international, 51:4283B.

[31] Ariyoshi T, Arakaki M, Ideguchi K, Ishizuka Y, Noda K, Ide H (1975). Studies on the metabolism of d-limonene (p-mentha-1,8-diene). III. Effects of d-limonene on the lipids and drug metabolizing enzymes in rat livers. Xenobiotica, 5:33-38. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

[32] Webb DR, Ridder GM, Alden CL (1989) Acute and subchronic nephrotoxicity of d-limonene in Fischer 344 rats. Food and chemical toxicology, 27:639-649. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

NCI Black Reiter rats, which do not synthesize alpha2μ-globulin, were dosed orally with d-limonene for 4 days at 1650 mg/kg bw/day and no effects on the kidney were seen (Dietrich, D.R. et al (1991)[33].Daily oral administration of 277-2770 mg/kg bw/day d-limonene to rats for one month caused slight decrease in body weight and food consumption. Histological examination of the kidneys revealed granular casts but there were no changes in other organs. (Tsuji, M. Et al 1975).[34]

Conclusion: No reasonable likelihood for repeated human exposure to QRD 460 is foreseen by the oral route. With limited occupational exposure and no exposure to residual product post-application, there is insignificant potential for repeated oral exposure to the marker components in QRD 460. The data on the active, formulation and one terpene component indicate low toxicity. Therefore, use of the active ingredient does not present any dietary or other oral toxicity issues and we believe there is no need for a 90-day sub-chronic feeding study**.**

B. Inhalation toxicity

End Product Use Pattern Does Not Trigger 90-day Inhalation Study: The representative formulation, QRD 452, contains 16.75% QRD 460 as the active ingredient. The intended uses of QRD 452 in Europe are commercial agricultural applications on plants intended for human consumption.

The following additional rationales further substantiate our contention that the intended use of the product is not likely to result in repeated human exposure by inhalation at levels likely to be toxic.

(a) Label Uses Minimise Exposure: The proposed label minimises the potential for human exposure. The limited application sites and application rate minimise the potential for significant repeated inhalation exposure by mixers/handlers and applicators and the general population. QRD 452 is neither labelled for, nor to be sold or marketed for use in homes, home gardens or other residential situations, which eliminates potential inhalation exposure of children and toddlers. Furthermore, the proposed application rate is 0.381 - 1.523 kg as/ha and QRD 452 will always be applied as a dilute solution in water, therefore, applicators will never be exposed to full strength product. At typical application volumes of 400 – 1000 litres of water per hectare this is equivalent to 38.1 – 381 g as/hL.

(b) Acceptable Safety for Operators, Workers and Bystanders EU models for assessing risk to operators, address both dermal and inhalation exposure though in most cases it is considered that dermal exposure will be the most important exposure route. In the case of QRD 460 all three terpenoid components are extremely volatile by nature and QRD 460 is likely to degrade rapidly in air to form smaller, naturally occurring molecules. For field applications it is considered that the rapid dissipation and dilution into the environment will mean that inhalation is not a significant exposure route. For the assessment of inhalation exposure in greenhouses the predicted environmental concentration PEC_{AIR} has been calculated as 0.087 mg/L. It should be noted that all evidence from modelling, the literature and anecdotal evidence suggests that none of the terpenoid constituents of QRD 460 persist in the air and are rapidly broken down. This means that the PEC_{AIR} value as calculated is worst case and any exposure is very short lived. Comparison of the PEC_{AIR} value of 0.087 mg/L with the acute inhalation LC_{50} for QRD 460 of >5.30 mg/L demonstrates a high margin of safety (2 orders of magnitude) for workers in glasshouses. Since the terpenoid constituents of QRD 460 do not persist in the air but are rapidly broken down potential for inhalation exposure will reduce rapidly. This is further supported by independent assessments conducted and evaluated for US approval (The Occupational Exposure and Risk Assessment for the Application of Extract of *Chenopodium ambrosioides* near *ambrosioides* to Row Crops report (# CE 07-02)[35] and MRID 47209102[36] contains an exposure scenario with air-blast

[33] Dietrich, D.R. & Swenberg, J.A. (1991a) NCI-Black-Reiter (NBR) male rats fail to develop renal disease following exposure to agents that induce α-2μ-globulin nephropathy. Fundam. appl. Toxicol., 16, 719-762. (Cited in IARC Monographs (1995). D-Limonene Volume 73)

[34] Tsuji, M., Fujisaki, Y., Arikawa, Y., Masuda, S., Kinoshita, S., Okubo, A., Noda, K., Ide, H. & Iwanage, Y. (1975a) [Studies on d-limonene, as gallstone solubilizer. II. Acute and subacute toxicities.] Oyo Yakuri, 9, 387-401 (in Japanese). (Cited in IARC Monographs (1995). D-Limonene Volume 73)

[35] E^{x}ponent®, Inc., Center for Chemical Regulation & Food Safety (2007a), Occupational Exposure and Risk Assessment for the Application of the Extract of *Chenopodium ambrosioides* near *ambrosioides* to Row Crops, Project Identification Number CE 07-02

sprayers). In both cases the reports show that when the appropriate personal protective equipment is utilized the margins of exposure (MOE) calculated for these types of applications methods are in excess of the target value, therefore, not of concern.

Lack of Acute Inhalation Toxicity: Studies on QRD 460 and QRD 452 indicate low acute inhalation toxicity to test animals (QRD 460 Inhalation LC_{50} rat > 5.32 mg/L, QRD 452 Inhalation LC_{50} rat > 5.19 mg/L). It is acknowledged that lack of acute toxicity is neither definitive nor necessarily predictive of sub-chronic toxicity. However, the measure of safety established by these studies contributes positively to the weight of evidence supporting this waiver request.

Conclusion: No reasonable likelihood for any meaningful level of repeated human exposure to QRD 460 is foreseen by the inhalation route. With limited occupational exposure, there is insignificant potential for repeated inhalation exposure to the terpene components in QRD 460 or QRD 452. Therefore, use of the active ingredient does not present any sub-chronic inhalation toxicity issues. Because the conditions for requiring such a study have not been met, we believe there is no need to conduct a 90-day subchronic inhalation study.

C. Percutaneous toxicity

End Product Use Pattern Does Not necessitate a 90-day dermal study: AQ's proposed end product, QRD 452, contains 16.75% QRD 460 as the active ingredient.

The intended uses of QRD 452 in Europe are commercial agricultural applications on plants intended for human consumption.

These uses do not involve the purposeful application to human skin nor will it result in prolonged human exposure to the product. Additionally, there is no evidence to suggest that the active ingredient is metabolised differently by the dermal route of exposure than by the oral route or that a metabolite of the active ingredient is the toxic moiety. More importantly, for several additional reasons outlined below, the intended use of the product is not likely to result in repeated human exposure by the dermal route.

Label Uses Minimise Exposure: The proposed label minimises the potential for human exposure in general, including the potential for repeated dermal exposure.

QRD 452 is neither labelled for, nor to be sold or marketed for use in homes, home gardens or other residential situations, which eliminates potential dermal exposure. Furthermore, the maximum proposed application rate is 1.523 kg as/ha and QRD 452 EC will always be applied as a dilute solution in water, therefore, applicators will never be exposed to full strength product.

Protective Clothing and Use Instructions: Label instructions specify that when using the product the following personal protective equipment and hygiene measures should be used:

Respiratory:	Not normally required in open, well-ventilated areas. However, in enclosed areas such as greenhouses/glasshouses, operators may be advised to wear respiratory protection.
Eyes and Face:	Chemical safety goggles or safety glasses with side shields.
Hands/Skin:	Gloves made from chemically resistant material such as neoprene, vinyl, rubber, or nitrile.
Other Clothing:	Wear suitable protective clothing such as long-sleeved shirt, trousers, and shoes with socks.
Hygiene Measures:	Wash hands and exposed skin before eating, drinking, smoking or using the toilet.

[36] E*x*ponent®, Inc., Center for Chemical Regulation & Food Safety (2007b), Occupational Exposure and Risk Assessment for the Application of the Extract of *Chenopodium ambrosioides* near *ambrosioides* (CE), Project Identification Number CE 07-01. (MRID 47209102)

Acceptable Safety for Operators, Workers and Bystanders. Risk assessments for operators, workers and bystanders have been conducted according to EU models to cover the proposed uses for products containing QRD 460. The models are based on many worse case assumptions and hence are conservative with the result that exposure is likely to be overestimated; however acceptable risk to operators, bystanders and workers can be demonstrated provided appropriate personal protective equipment is used. This conclusion is further supported by an independent occupational exposure assessment for typical greenhouse and agricultural row crop applications for the blended active ingredient formulated product, QRD 452 conducted for approval in the US. The report (Report ID: ECANA Mimic 10-01)[37] demonstrates that QRD 452 may be applied in greenhouses and to row crops without any cause for concern as long as appropriate personal protective equipment – long sleeve shirt, long trousers, shoes plus socks, and water resistant gloves – is worn. The total margin of exposure (MOE) for all mixer/loader and applicator scenarios is greater than the target value of 100. Occupational re-entry exposures will be negligible, since several residue decline studies have demonstrated that the active ingredient residues do not persist on foliar surfaces beyond the time required for the spray to dry. In other words, immediate dermal exposure from handling or applying the

Rapid Foliar Residue Decline Residues of all three terpene components of the active substance declined to non-detectable levels within 10 minutes after foliar applications of Facin 25EC on Primrose at an application rate of 9.35 L as/ha. (Note: FACIN 25EC is a discontinued brand name which was replaced with the name Requiem 25EC, the plant extract-based product) Thus, the potential for any post-application dermal exposure by applicators or others is essentially non-existent. AQ conducted a residue decline study titled Persistence of Facin 25% EC on Primrose (*Primula acaulis*) (MRID 47209101, see Document MII Section 4 for full details), which shows rapid dissipation of the Facin components, thus supporting the argument that exposure to residual product is also of minimal concern. The study clearly showed that even at an exaggerated application rate, the three marker components are not detectable within 10 minutes of product application. Essentially, by the time the leaves have dried there is no detectable residual product. This limits the window of opportunity for dermal exposure from foliage after application. The requested 4-hour Re Entry Interval further expands the safety margin for this product.

Results of the Primrose study are consistent with and supported by the residue decline study Agraquest Inc. conducted using Facin 25EC on tomatoes (MRID 46858903, see Document MII Section 4 for full details). The tomato study demonstrated rapid dissipation of the active constituents of the extract of *Chenopodium ambrosioides*, at 0, 3, 6, 12, and 24 hr post-treatment. All constituents were less than the limit of quantitation (<LOQ) of 0.01 mg/kg at all-time intervals except for d-limonene, which was attributed to background d-limonene naturally occurring in tomatoes. The product was applied four times using an application rate of ~2 kg as/ha. In summary, results of both the Primrose study and the Tomato study demonstrate that multiple applications of Facin 25EC results in no detection of residue shortly after application and no accumulation of residues over multiple applications.

Finally, results of a residue decline study performed for AgraQuest Inc. with the QRD 452 product (then called QRD 416) showed that residues of the three major terpene components declined to non-detectable levels within one hour after foliar application on mustard greens at rates of 2690 – 2914 g as/ha. In the study, QRD 400/QRD 416: Residue Levels of Terpenes in Mustard Greens from a Trial Conducted in California during 2007 (Study ID No. 77SRU07R-1, MRID 47548301, see Document MII Section 4 for full details) the product was applied three times at specified intervals and the plants were sampled at 0, 1, and 4 hours after the third application.

Lack of Acute Dermal Toxicity. Studies on QRD 460 and QRD 452 establish that neither product is highly acutely toxic to mammals. The dermal LD_{50} in rats was greater than 5,050 mg/kg and 2,020 mg/kg in studies with the technical and formulated products, respectively. Whilst the lack of acute toxicity is neither definitive nor necessarily predictive of sub-chronic toxicity, the measure of safety established by these studies contributes positively to the weight of evidence supporting this waiver request.

Conclusion: There is no reasonable likelihood for repeated human exposure to QRD 460 by the dermal route. With limited occupational exposure and no exposure to residual product post-application, there is insignificant potential for repeated dermal exposure to the terpene components in QRD 452. Therefore, use of the active ingredient does not present any dermal toxicity issues and there is no need for a 90-day sub-chronic dermal study.

[37] E^x^ponent®, Inc., Center for Chemical Regulation & Food Safety (2010), Occupational Exposure and Risk Assessment for the Application of QRD 452 in Greenhouses and to Row Crops (Metric Units), Project Identification Number ECANA Mimic 10-01.

IIA 5.3.1 Oral 28-day toxicity

See section IIA 5.3 above.

IIA 5.3.2 Oral 90-day toxicity (rodents)

See section IIA 5.3 above.

IIA 5.3.3 Oral 90-day toxicity (dog)

See section IIA 5.3 above.

IIA 5.3.4 Oral 1 year toxicity (dog)

See section IIA 5.3 above.

IIA 5.3.5 28-day inhalation toxicity (rodents)

See section IIA 5.3 above.

IIA 5.3.6 90-day inhalation toxicity (rodents)

See section IIA 5.3 above.

IIA 5.3.7 Percutaneous 28-day toxicity (rodents)

See section IIA 5.3 above.

IIA 5.3.8 Percutaneous 90-day toxicity (rodents)

See section IIA 5.3 above.

IIA 5.4 Genotoxicity

QRD 406 or its main components have been examined in three genotoxicity assays; bacterial reverse mutation assay, mammalian chromosome aberration test and an unscheduled DNA repair assay.

QRD 406 is the code number given to Chenopodium plant extract technical grade active ingredient (tgai). The active components are the three terpenes (α-terpinene, ρ-cymene, d-limonene) in similar proportions as in QRD 460 and hence it is considered that results obtained from studies with QRD 406 are also applicable to QRD 460. Full compositional details are provided in Document J as this information is confidential.

Table IIA 5.4-1: QRD 406 - Summary of genotoxicity studies

Study	Dose Levels	Result	Reference
In vitro studies			
Bacterial reverse mutation (OECD 471)	0-190 µg/plate	Negative	[redacted] ***B, 2003***
Mammalian chromosome aberration (OECD 473)	0-1.4 mM	Negative	[redacted] ***A, 2004***
Unscheduled DNA repair assay (OECD 482)	0.00005 mM - 0.158 mM	Negative	[redacted] ***L, 2004***

QRD 406, containing significant levels of all three relevant terpenes, has been evaluated for possible genotoxic activity in three in vitro assays, covering three different endpoints. The Ames test for gene mutation, the IVC assay for clastogenic effects and the UDS assay for general DNA damage and repair activity. In all three assays QRD 406 was examined at doses limited by toxicity, and in the Ames and IVC assays, in both the absence and presence of auxiliary metabolic activation. The use of the Ames test and the IVC assay are considered key assays for examining for any possible genotoxic activity arising from the alkene components present in QRD 406, and the conduct of the usual mammalian cell gene mutation assay would not have added any significant additional information. The UDS assay, however, adds an evaluation for general DNA damage/repair and this is valuable. Chemicals containing simple alkene groups would be expected to be active in *in vitro* assays if they are genotoxic, and it is considered that there is no justification for using animals for an additional investigation, given the negative results obtained from the three assays above.

The negative results obtained with QRD 406 are consistent with a Structure Activity Relationship[38] (SAR) evaluation, which only alerts to possible active centres in the alkene groups for limonene and terpinene. The available metabolic data for limonene indicates that metabolism is to chemicals that are readily excreted, and would not be expected to show significant genotoxic activity.

Literature Sources: The above is supported by published genotoxicity data on all three QRD460 components.

d-Limonene has been studied in a battery of short-term *in vitro* tests, there is no evidence that the material or its metabolites are genotoxic[39]. Several *in vitro* Ames studies are available which report d-limonene and its epoxides being tested at concentrations of 0.3-3333 µg/plate in in vitro assays in different strains of *Salmonella typhimurium,* in the presence or absence of metabolic activation (Florin *et al*., 1980[40]; Watabe *et al*., 1981[41]; Haworth *et a*l.,1983[42]; Connor *et al*., 1985[43]; NTP, 1990[44]). All were negative. NTP, 1990 report d-limonene did not increase the frequency of forward mutation at the TK+/- locus in mouse L5178Y cells, it did not induce cytogenetic damage in Chinese hamster ovary cells (Anderson et al., 1990[45]), or malignantly transform Syrian hamster embryo cells (Pienta, 1980[46]). Similarly no evidence of mutagenicity was reported in an *in vivo* spot test with mice (limonene administered via the i.p. route at 215 mg/kg bw/day on days 9-11 during gestation (Fahrig, 1984[47])or an invivo Comet assay in rats and mice (dosed orally at 2000 mg/kg) (Sekihashi *et al* 2002)[48]

[38] Tennant RW and Ashby J (1991). Classification according to chemical structure, mutagenicity to Salmonella and level of carcinogenicity of a further 39 chemicals by the US National Toxicology Program. Mutat Res 257 (3) 209-227

[39] IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene

[40] Florin I, Rutberg L, Curvall M, Enzell CR (1980) Screening tobacco smoke constituents for mutagenicity using the Ames test. Toxicology, 18:219-232. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene

[41] Watabe T, Hiratsuka A, Osawa N, Isobe M (1981) A comparative study on the metabolism of d-limonene and 4-vinylcyclohex-1-ene by hepatic microsomes. Xenobiotica, 11:333-344. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

[42] Haworth S, Lawlor T, Mortelmans K, Speck W, Zeiger E (1983) Salmonella mutagenicity test results for 250 chemicals. Environmental mutagenesis, 1:3-142. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

[43] Connor TH, Theiss JC, Hanna HA, Monteith DK, Matney TS (1985). Genotoxicity of organic chemicals frequently found in the air of mobile homes. Toxicology letters, 25:33-40. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

[44] NTP (1990). Toxicology and carcinogenesis studies of d-limonene in F344/N rats and $B6C3F_1$ mice. Springfield, VA, US Department of Health and Human Services, National Institutes of Health, National Toxicology Program (NTP Technical Report No. 347). (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

[45] Anderson BE, Zeiger E, Shelby MD, Resnick MA, Gulati DK, Ivett JL, Loveday KS (1990) Chromosome aberration and sister chromatid exchange test results with 42 chemicals. Environmental and molecular mutagenesis, 16:55-137. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

[46] Pienta RJ (1980) Evaluation and relevance of the Syrian hamster embryo cell system. Applied methods in oncology, 3:149-169. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

[47] Fahrig R (1984). Genetic mode of action of cocarcinogens and tumor promoters in yeast and mice. Molecular & general genetics, 194:7-14. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

[48] Sekihashi K et al (2002) Comparative investigation of multiple organs of mice and rats in the Comet assay. Mutat Res.517 (1-2) 53-75.

One genotoxicity study on α-terpinene was identified reported by Gomes-Carneiro *et al*. (2005)[49]. Mutagenicity was evaluated by the Salmonella/microsome assay (strains TA100, TA98, TA97a and TA1535), with and without metabolic activation. A broad range of doses were tested at levels limited by toxicity and the results indicated α-terpinene was not mutagenic in this assay.

p-Cymene is reported by Rockwell and Wall (1979) as testing negative in an Ames assay using Salmonella typhimurium strains TA98 and TA100. 0.5 ml (reported as equivalent to 1706mg/kg bw) was "administered" to Sprague Dawley rats, the urine was collected and tested in vitro[50].

IIA 5.4.1 *In vitro* genotoxicity testing - Bacterial assay for gene mutation

Report:	IIA 5.4.1/01 [redacted] B, 2003. Bacterial reverse mutation test of 03D-2. [redacted]. Laboratory Report No. 120940, 29 August 2003. Unpublished.

Guidelines

Reverse Mutation Test Using Bacteria. OPPTS 870.5100 US EPA 712-C-98-247 (1998):

GLP: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

Executive Summary

QRD 406 (containing >99,9% monoterpenes (the main ones were alpha-terpene, *p*-cymene, *d*-limonene and *p*-menth-2-ene-1,4-diol) was tested in a bacterial reverse mutation assay for its potential to induce point mutations in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and *E. coli* strain WP2 *uvrA*.

The potential mutagenicity of QRD 406 was tested in the presence and absence of metabolic enzymes, S9, from livers of rats treated with Aroclor 1254. Experiment 1 was a plate incorporation assay and experiment 2 was a preincubation assay. Exposure concentrations were 0, 2.3, 7.0, 21.0, 63.0 and 190 µg per plate. Triplicate plates were included for each concentration.

No precipitation of the test substance was observed on the plates at any exposure concentration before or after incubation.

Signs of bacterial toxicity were clearly observed at the maximum exposure concentration, 190 µg per plate, demonstrated in all tester strains by various degrees of reduction in the background bacterial lawn. Exposure to all concentrations below the maximum exposure concentration resulted in a background level of reversion with minimal bacterial toxicity. The numbers of revertants per plate from these non-toxic concentrations were largely within the range of the means ± 2 standard deviations (SDs) of the corresponding solvent controls. They were all below the upper limit of the respective historical dimethylsulfoxide control. These negative results were consistent between plate incorporation and preincubation studies.

It was therefore concluded that QRD 406 (>99.9% monoterpenes) was not mutagenic to *S. typhimurium* strains, TA98, TA100, TA1535, TA1537, and *E. coli* strain, WP2 *uvrA*, under the test conditions.

[49] Gomes-Carneiro MR, Viana ME, Felzenszwalb I, Paumgartten FJ (2005).Evaluation of beta-myrcene, alpha-terpinene and (+)- and (-)-alpha-pinene in the Salmonella/microsome assay. Food Chem Toxicol. Feb;43(2):247-52

[50] Rockwell P and Raw I (1979). A mutagenic screening of various herbs, spices and food additives. Nutr. Cancer 1 (4), 10-15. Cited in Opinion of the Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC) related to Flavouring Group Evaluation 18 (FGE.18): Aliphatic, alicyclic and aromatic saturated and unsaturated tertiary alcohols, aromatic tertiary alcohols and their esters from chemical group 6 (2006). The EFSA Journal 331, 1-77.

Materials:

Test Material: QRD 406
Description: Light yellow liquid, extracted from a herbaceous plant
Lot/Batch number: 03D-2, Lot C
Purity: >99.9% of the sample was monoterpenes (the main ones were alpha-terpene, *p*-cymene, *d*-limonene and *p*-menth-2-ene-1,4-diol.)
CAS#: Not reported
Stability of test compound: Expiry date 23 April 2004

Control Materials:
Vehicle control: Dimethylsulfoxide (DMSO)
Positive control: Nonactivation:
Sodium azide 5 µg/plate TA100, TA1535
Methyl methanesulfonate 1 µL/plate WP2 *uvrA*
2-nitrofluorene 5 µg/plate TA98
9-aminoacridine hydrochloride 80 µg/plate TA1537
Activation:
2-Aminoanthracene 100 µg/plate TA1537, 100 µg/plate WP2 *uvrA*
Benzo (a)pyrene 5.0 µg/plate TA98, TA100, TA1537
Cyclophosphamide monohydrate 100 µg/plate TA1535

Mammalian metabolic system: S9 derived

X	Induced	X	Aroclor 1254	X	Rat	X	Liver
	Non-induced		Phenobarbitol		Mouse		Lung
			None		Hamster		Other

S9 was purchased from Molecular Toxicology Inc., Boone, NC, U.S.A. and stored at -80°C. It was the 9,000 x g fraction of liver homogenate from Sprague-Dawley rats treated with Aroclor 1254. Immediately prior to use, an S9 mix was constituted by the standard formula (Maron and Ames, 1983): 0.1 M NaH_2PO_4 / Na_2HPO_4, pH 7.4; 5 mM Glucose-6-phosphate; 4 mM NADP; 33 mM KCl; 8 mM $MgCl_2$ and 10% rat liver S9.

Test organisms:

***S. typhimurium* strains**

	TA97	X	TA98	X	TA100		TA102		TA104
X	TA1535	X	TA1537		TA1538		list any others		

***E. coli* strains**

	WP2 (pKM101)	X	WP2 *uvrA*						

Properly maintained? [X] Yes [ ] No

All strains were purchased as dried discs and stored in a refrigerator. Frozen permanent stocks were prepared from fresh cultures of the discs and stored at -80°C after the addition of 9% dimethylsulfoxide.

Test compound concentrations used:

Exposure concentrations were 0, 2.3, 7.0, 21.0, 63.0 and 190 µg/plate.

For all strains, triplicate plates were used for all test substance and positive control and vehicle control treatments.

Study Design and Methods:

In-life dates: Start: 22 July 2003 End: Not reported

Preliminary Cytotoxicity Assay (plate incorporation): Method not reported.

Type of Bacterial assay:

- X plate incorporation assay (-S9 and +S9)
- X pre-incubation (30 minutes) (-S9 and + S9)

Protocol: 0.1 mL of a test substance solution was mixed with 0.1 mL of a fresh overnight culture and 0.5 mL of S9 mix or 0.5 mL of 0.1 M phosphate buffer, pH 7.4.

In the plate incorporation assay, this mixture was incorporated into 2 mL top agar by that sequence and poured onto the bottom agar immediately.

In the preincubation assay, the mixture was pre-incubated at 30°C for 30 min before mixing with the top agar. The control tubes and plates were handled in a manner identical to the treated ones.

All plates were incubated at 37±2°C for 48-72 hours. The number of revertant colonies per plate was then counted. The background lawn was evaluated as part of the evidence of toxicity. Precipitation of the test substance was also examined.

Statistical analysis: Dunnett's analysis was used to compare data from the treated and DMSO-control plates.

Evaluation criteria:
Positive result: A concentration-related increase over the range tested and a reproducible increase in at least one or more concentrations in the number of revertant colonies per plate in at least one strain with or without metabolic activation system. A statistical method may be used as an aid in evaluating the test results but it should not be the only determining factor. A positive result indicates that the test article induces point mutations in *S. typhimurium* or *E. coli.*

Negative result: If a result does not meet the above criteria, it will be reported as negative. A negative result indicates that the test article is non-mutagenic in this test.

Equivocal result: If no definite judgement can be made to fit the above criteria, even after repeated experiments, then the result will be described as equivocal.

Reported Results

Preliminary plate incorporation assay: Bacterial toxicity of QRD 406 was demonstrated as a reduced background bacterial lawn and a decreased number of revertants per plate. QRD 406 showed concentration-related toxicity to TA 100 and WP2 uvrA. At concentrations below 63 µg per plate, little toxicity was detected in either strain, with or without the presence of S9. At 190 µg per plate, a slight visible reduction of the background bacterial lawn was observed in TA100 plates and a very slight level of reduction was seen microscopically in WP2 uvrA plates.

The addition of S9 did not seem to affect the bacterial toxicity of the test substance. At 560, 1,667 and 5,000 µg per plate, the bacterial lawn of TA100 plates was markedly or completely abolished with or without S9 while the lawn of WP2 uvrA plates grew into clearly visible colonies.

Experiment 1 (plate incorporation assay). As predicted by the results of the preliminary study, 03D-2 was toxic to the tester strains at the maximum exposure concentration, 190 µg per plate. A slight but clear reduction of the background bacterial lawn was observed in all *S. typhimurium* strains whereas, in WP2 uvrA, a reduction was seen only with the aid of a microscope. In a few plates, the lawn bacteria that escaped the toxic effect of the test substance overgrew into visible colonies indistinguishable from the revertant colonies.

No obvious toxic signs were observed at four lower concentrations. The numbers of revertants per plate at these concentrations were at a level similar to the corresponding solvent controls. The mean revertant numbers for triplicate plates were largely below the means plus 2 SDs of the respective DMSO controls. An exception was TA100. In the presence of S9, the numbers of revertants per plate at 2.3, 7.0, 63 and 190 µg per plate were 148±14,

161±15, 149±8, and 150±7, revertant colonies per plate, respectively, slightly above the mean plus 2 SD of the DMSO control, (126+8). Although the differences were significant by one-sided Dunnett's analysis ($p < 0.05$), these means showed no trend to increase with higher exposure concentrations of 03D-2. The data was, therefore, not treated as a positive result and not analyzed any further.

Experiment 2 (preincubation assay). The reversion rate of every tester strain after exposure to 03D-2 was at a level of the corresponding negative control. One mean of WP2 uvrA plates without S9 at 2.3 µg per plate was 28±10 revertants per plate, slightly above its negative control 16±4. Similarly, the mean of TA1537 plates with S9 at 63 µg per plate was 15±3 revertant colonies per plate, marginally over the mean plus 2 SD of its DMSO control. Dunnett's analysis showed no significance in both cases ($p < 0.05$). Besides these exceptions, the numbers of revertants per plate from the rest of experiment 2 were below the means plus 2 SDs of the corresponding negative controls.

All the concurrent positive controls in the experiments induced significant numbers of revertant colonies, indicating that the sensitivity of the assay was at a reasonable level. The concurrent negative controls were at similar levels of the respective historical negative controls.

Conclusion

QRD 406 was not mutagenic to *S. typhimurium* strains TA98, TA100, TA1535, TA1537, and *E. coli* strain, WP2 *uvrA*, under the test conditions.

Reference:

Maron, D.M. and Ames, B.N., Revised methods for the Salmonella mutagenicity test. *Mutat. Res.*, 113:173-215, 1983

([redacted] B, 2003)

IIA 5.4.2 *In vitro* genotoxicity testing - Test for clastogenicity in mammalian cells

Report:	IIA 5.4.2/01 [redacted] A, 2004. Facin: *In vitro* mammalian chromosome aberration test in human lymphocytes. [redacted]. Laboratory Report No LDA/061/043212, 18 October 2004. Unpublished

Guidelines

In Vitro Mammalian Chromosome Aberration Test. OECD 473 (1997): OPPTS 870.5375 (1998): 2000/32/EC B10 (2000), ICN Guideline S2A (1996): ICN Guideline S2B (1998)

GLP: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

Executive Summary

In a mammalian cell cytogenetics assay, human lymphocytes in whole blood culture, were stimulated to divide by addition of phytohaemagglutinin, and exposed to Facin (100% a.i, QRD 406) both in the presence and absence of S9 mix derived from rat livers. Solvent and positive control cultures were also prepared. Two hours before the end of the incubation period, cell division was arrested using Colcemid®, the cells harvested and slides prepared, so that metaphase cells could be examined for chromosomal damage.

In order to assess the toxicity of Facin to cultured human lymphocytes, the mitotic index was calculated for all cultures treated with the test substance and the solvent control. On the basis of these data, the following concentrations were selected for metaphase analysis:

First test: Without S9 mix - 3 hours treatment, 17 hours recovery: 0.25, 0.75 and 1.0 mM. With S9 mix - 3 hours treatment, 17 hours recovery: 0.7, 1.1 and 1.4 mM.

Second test: Without S9 mix - 20 hours continuous treatment: 0.1, 0.2 and 0.4 mM. With S9 mix - 3 hours treatment, 17 hours recovery: 1. 0, 1.2 and 1.4 mM.

In both the absence and presence of S9 mix, Facin caused no statistically significant increases in the proportion of metaphase figures containing chromosomal aberrations, at any dose level, when compared with the solvent control, in either test.

All positive control compounds caused large, statistically significant increases in the proportion of aberrant cells, demonstrating the sensitivity of the test system and the efficacy of the S9 mix.

It is concluded that Facin (QRD 406) has shown no evidence of clastogenic activity in this *in vitro* cytogenetic test system, under the experimental conditions described.

Materials:

Test Material:	Facin
Description:	Essential oil extract of *Chenopodium ambrosioides var ambrosioides*. Mixture of several monoterpenes (majority of compounds) and sesquiterpenes. Pale yellow and transparent liquid
Lot/Batch number:	03D-2e
Purity:	100%
CAS#:	Not reported
Stability of test compound:	Expiry date 30 September 2005

Control Materials:	
Negative:	Sterile purified water
Solvent control (final concentration):	10 mM
Positive control:	Absence of S9 mix: Mitomycin C 0.2 µg/mL (3 hour treatment), 0.1 µg/mL (continuous treatment) Presence of S9 mix: Cyclophosphamide 10µg/mL

Mammalian metabolic system: S9 derived

X	Induced	X	Aroclor 1254	X	Rat	X	Liver
	Non-induced		Phenobarbitol		Mouse		Lung
			None		Hamster		Other
			Other β-naphthoflavone		Other		

The metabolic activation system (S9-mix) used in this study was prepared and stored at -80°C or below until required. S9 mix contained: S9 fraction (10% v/v), $MgCl_2$ (8 mM), KCl (33 mM), sodium phosphate buffer pH 7.4 (100 mM), glucose-6-phosphate (5 mM), NADP (4 mM). All the cofactors were filter-sterilised before use.

Test cells: mammalian cells in culture

	V79 cells (Chinese hamster lung fibroblasts)
X	Human lymphocytes.
	Chinese hamster ovary (CHO) cells
Media	RPMI-1640

Test compound concentrations used:

First test	
Absence of S9 mix	0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00 and 2.25 mM
Presence of S9 mix	0.50, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25 and 2.50 mM
Due to inadequate toxicity in the presence of S9 a further repeat test was necessary to isolate suitable levels of cytotoxicity.	0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3 and 1.4 mM
Second test	
Absence of S9 mix	1.4, 1.2, 1.0, 0.8, 0.6, 0.4, 0.2, 0.1, 0.05 and 0.025 mM
Presence of S9 mix	1.6, 1.4, 1.2, 1.0, 0.8, 0.6, 0.4 and 0.2 mM

Study Design and Methods:

In-life dates: Start: 28 June 2004 End: 27 August 2004

Experimental Procedure

Culture of lymphocytes: Human blood was collected aseptically from 2 healthy, non-smoking male donors, pooled and diluted with RPMI 1640 tissue culture medium supplemented with 10% foetal calf serum, 1 I.U/mL sodium heparin, 20 I.U/mL penicillin / 20 µg/mL streptomycin and 2.0 mM glutamine. Aliquots of the cell suspension were incubated at 37°C for approximately 48 hours. The cultures were gently shaken daily to resuspend the cells.

Treatment of cells – first test: After approximately 48 hours, 50 µL aliquots of Facin were added to one set of duplicate cultures to give final concentrations of 0.078, 0.156, 0.313, 0.625, 1.25, 2.50, 5.00 and 10.0 mM. DMSO, the solvent control, in 50 µL aliquots, was added to two cultures. Mitomycin C, at a final concentration of 0.2 µg/mL, was added to duplicate cultures.

Immediately before treatment of the second set of cultures, 1 mL of medium was removed from each culture and discarded. This was replaced with 1 mL of S9 mix, followed by 50 µL aliquots of the various dilutions of Facin, giving the same series of final concentrations as above. DMSO (50 µL) was added to two cultures. Cyclophosphamide was added to duplicate cultures at a final concentration of 10 µg/mL.

Three hours after dosing, the cultures were centrifuged at 500 g for 5 minutes. The cell pellets were rinsed and resuspended in fresh medium. They were then incubated for a further 17 hours.

Due to a steep toxic response in the absence and presence of S9 mix, the test was repeated in order to isolate levels of suitable cytotoxicity for metaphase analysis.

Two hours before the cells were harvested, mitotic activity was arrested by addition of Colcemid® to each culture at a final concentration of 0.1 µg/mL. After 2 hours incubation, each cell suspension was transferred to a centrifuge tube and centrifuged for 5 minutes at 500 g. The cell pellets were treated with a hypotonic solution 0.075M (KC1) (pre-warmed at 37°C). After a 10 minute period of hypotonic incubation at 37°C, the suspensions were centrifuged at 500 g for 5 minutes and the cell pellets fixed by addition of freshly prepared cold fixative (3 parts methanol : 1 part glacial acetic acid). The fixative was replaced twice.

The pellets were resuspended, then centrifuged at 500 g for 5 minutes and finally resuspended in a small volume of fresh fixative. A few drops of the cell suspensions were dropped onto pre-cleaned microscope slides which were then allowed to air-dry. The slides were then stained in 10% Giemsa, prepared in buffered water (pH 6.8). After rinsing in buffered water the slides were left to air-dry and then mounted in DPX. The remaining cultures in fixative were stored at 4°C until slide analysis was completed.

Treatment of cells – second test: Cultures were initiated and maintained as previously described. In this second test a continuous treatment was used in the absence of S9 mix. In the presence of S9 mix, a three hour treatment was used, as in the first test. The harvest time was at 20 hours for both parts of the test.

Duplicate cultures were used for each treatment and two cultures were treated with the solvent control. Positive control cultures were treated as in the first test. Mitomycin C, at a final concentration of 0.1 µg/mL, and Cyclophosphamide, at a final concentration of 10 µg/mL, were added to duplicate cultures.

Three hours after dosing, the cultures containing S9 mix were centrifuged. The cell pellets were rinsed and resuspended in fresh medium. They were then incubated for a further 17 hours. Cultures treated in the absence of S9 mix were incubated for 20 hours.

All cultures were treated with Colcemid®, at a final concentration of 0.1 µg/mL, two hours before the end of the incubation period. They were then harvested, fixed and the slides prepared and examined as previously described.

Evaluation criteria:

An assay is considered to be acceptable if the negative and positive control values lie within the current historical control range.

The test substance is considered to cause a positive response if the following conditions are met:

- Statistically significant increases ($P<0.01$) in the frequency of metaphases with aberrant chromosomes (excluding gaps) are observed at one or more test concentration.
- The increases exceed the negative control range of this laboratory, taken at the 99% confidence limit.
- The increases are reproducible between replicate cultures.
- The increases are not associated with large changes in pH, osmolality of the treatment medium or extreme toxicity.
- Evidence of a dose-relationship is considered to support the conclusion.

A negative response is claimed if no statistically significant increases in the number of aberrant cells above concurrent control frequencies are observed, at any dose level.

A further evaluation may be carried out if the above criteria for a positive or a negative response are not met.

Statistical analysis: The number of aberrant metaphase cells in each treatment group was compared with the solvent control value using the one-tailed Fisher exact test (Fisher 1973).

Reported Results

Toxicity data: In the first test, due to inadequate toxicity profiles, repeat tests were performed in order to isolate levels of suitable cytotoxicity for metaphase analysis. In the absence of S9 mix, Facin caused a reduction in the mitotic index to 45% of the solvent control value at 1.0 mM. The dose levels selected for the metaphase analysis were 0.25, 0.75 and 1.0 mM. In the presence of S9 mix, Facin caused a reduction in the mitotic index to 48% of the solvent control value at 1.4 mM. The dose levels selected for the metaphase analysis were 0.7, 1.1 and 1.4 mM.

In the second test, in the absence of S9 mix, Facin caused a reduction in the mitotic index to 61% of the solvent control value at 0.4 mM. The dose levels selected for the metaphase analysis were 0.1, 0.2 and 0.4 mM. In the presence of S9 mix, Facin caused a reduction in the mitotic index to 49% of the solvent control value at 1.4 mM. The dose levels selected for the metaphase analysis were 1.0, 1.2 and 1.4 mM.

Metaphase analysis: In both tests, in both the absence and the presence of S9 mix, Facin caused no statistically significant increases in the proportion of cells with chromosomal aberrations at any dose level, when compared with the solvent control.

Both positive control compounds, Mitomycin C and Cyclophosphamide, caused large, statistically significant increases ($P<0.001$) in the proportion of aberrant cells. This demonstrated the efficacy of the S9 mix and the sensitivity of the test system.

Table IIA 5.4.2-1: Summary of results – test 1

Exposure period (hours)	S9 mix	Concentration of Facin (mM)	Cells with aberrations, excluding gaps			Cells with aberrations, including gaps			Relative mitotic index (%)
			Individual values (%)		Mean (%)	Individual values (%)		Mean (%)	
3	-	0 (DMSO)	1	1	1.0	1	1	1.0	100
		0.25	0	2	1.0	0.2	2	1.0	80
		0.75	2	1	1.5	2	1	1.5	75
		1.0	0	2	1.0	0	3	1.5	45
		0.2 µg/mL Mitomycin C	17	14	15.5***	18	15	16.5***	-
3	+	0 (DMSO)	2	1	1.5	2	1	1.5	100
		0.7	4	3	3.5	5	3	4.0	104
		1.1	0	1	0.5	0	1	0.5	71
		1.4	4	3	3.0	6	2	4.0	48
		0.2 µg/mL cyclophosphamide	28	24	26.0***	30	27	28.5***	-

*** = p<0.001 otherwise p≥0.01

Table IIA 5.4.2-1: Summary of results – test 2

Exposure period (hours)	S9 mix	Concentration of Facin (mM)	Cells with aberrations, excluding gaps			Cells with aberrations, including gaps			Relative mitotic index (%)
			Individual values (%)		Mean (%)	Individual values (%)		Mean (%)	
20	-	0 (DMSO)	0	1	0.5	0	1	0.5	100
		0.1	1	1	1.0	2	2	2.0	80
		0.2	2	3	2.5	2	3	2.5	75
		0.4	1	1	1.00	1	2	1.5	61
		0.1 µg/mL Mitomycin C	12	15	13.5***	14	17	15.5***	-
3	+	0 (DMSO)	1	1	1.0	1	2	1.5	100
		1.0	2	1	1.5	2	2	2.0	86
		1.2	3	2	2.5	3	2	2.5	68
		1.4	0	2	1.0	2	3	2.5	49
		10 µg/mL cyclophosphamide	15	18	16.5***	17	19	18.0***	-

*** = p<0.001 otherwise p>0.01

Conclusion

It is concluded that Facin has shown no evidence of clastogenic activity in this in vitro cytogenetic test system, under the experimental conditions described.

Reference:

Fisher RA. The Exact Treatment of 2 x 2 Table in: *Statistical Methods for Research Workers*. Hafner Publishing Company, New York. 1973

([redacted]A, 2004)

IIA 5.4.3 *In vitro* genotoxicity testing - Test for gene mutation in mammalian cells

Report:	IIA 5.4.3/01 [redacted] L, 2004. Facin: In vitro DNA repair (UDS) test using rat hepatocytes. [redacted]. Laboratory Report No. LDA 062/043249, 08 October 2004. Unpublished.

Guidelines

Unscheduled DNA Synthesis (UDS) in Mammalian Cells *In vitro*: OECD 482 (1986); OPPTS 870.5550 (1998); EPA 712-C-98-230

GLP: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

Executive Summary

Facin (QRD 406) (purity 100%) was tested for potential mutagenic activity by measuring its ability to cause DNA repair in cultured rat hepatocytes on two independent occasions.

For each test, hepatocytes were isolated by enzymatic dissociation from one male CD rat and were cultured *in vitro*. The isolated hepatocytes were allowed to attach to coverslips and were treated with either the solvent control, test substance or positive control for 18 hours together with (methyl-^{3}H) thymidine at a final concentration of 10 µCi/mL to 'radiolabel' DNA undergoing repair replication.

All cultures were treated with a dose volume of 20 µL per 2 mL well. Solvent control cultures were treated with the solvent, dimethylsulphoxide (DMSO). Positive control cultures were treated with 2-acetylaminofluorene (2-AAF).

Following treatment, the hepatocytes were 'chased' for 24 hours with media containing unlabelled thymidine, they were then fixed and processed for autoradiography.

The incorporated radioactive thymidine results in the deposition of silver grains in the overlying autoradiographic emulsion. After fixation and development, toxicity was assessed and the seven highest concentrations of cultures treated with the test substance showing no toxicity were selected for scoring as follows:

First test: 0. 05, 0.0158, 0.005, 0.00158, 0.0005, 0.000158 and 0.00005 mM

Second test: 0.158, 0.05, 0.0158, 0.005, 0.00158, 0.0005, and 0.000158 mM

DNA repair was assessed by comparing the grain count of hepatocyte nuclei with the accompanying cytoplasmic grain count. The gross nuclear grain count and the net nuclear grain count from test substance treated cultures were compared with the concurrent vehicle control values.

No statistically significant increases in the net nuclear grain count, cytoplasmic grain count or gross nuclear grain count were obtained at any dose level of Facin in either test.

Cultures treated with the positive control agent (2-AAF) showed a statistically significant increase in the net nuclear grain count which was accompanied by an increase in the gross nuclear grain count.

It is concluded that Facin (QRD 406) did not shown any evidence of causing unscheduled DNA synthesis in this *in vitro* test system.

Materials:

Test Material:	Facin
Description:	Mixture of several monoterpenes (majority of compounds) and sesquiterpenes. Pale yellow and transparent liquid
Lot/Batch number:	03D-2e
Purity:	100% a.i
CAS#:	Not reported
Stability of test compound:	Expiry date: 30 September 2005

Control Materials:

Vehicle:	dimethylsulphoxide (DMSO)	**Dose volume:**	20 µL per 2 mL well
Positive control :	2-Acetylaminofluorene (2-AAF)	**Dose volume:**	20 µL per 2 mL well

Study Design and Methods:

In-life dates: Start: 20 July 2004 End: 17 September 2004

UDS Assay: The study consisted of two independent tests to assess the potential of the test substance to cause DNA damage in rat liver cells.

The seven highest concentrations of cultures treated with the test substance showing no toxicity were selected for scoring as follows:

First test: 0. 05, 0.0158, 0.005, 0.00158, 0.0005, 0.000158 and 0.00005 mM

Second test: 0.158, 0.05, 0.0158, 0.005, 0.00158, 0.0005, and 0.000158 mM

Cell Preparation: Immediately prior to use, hepatocytes were isolated from a Sprague-Dawley male rat.

The animal was killed by exposure to an increasing concentration of carbon dioxide. The liver was exposed and the hepatic portal vein was cannulated. Perfusing media were held in a water bath at approximately 43°C to give a temperature of approximately 37°C at the outlet. The liver was initially perfused with EGTA solution for 3 minutes to deplete the liver of calcium ions and reduce cellular adhesion. The vena cava was cut below the liver to relieve any excess pressure on the liver to allow the perfusate to drain through. The liver was perfused with collagenase solution for 10 minutes, excised and placed in a petri dish with a further aliquot of collagenase solution. The liver cells were combed into suspension then filtered through nylon bolting cloth (200 µm mesh).

The hepatocyte suspension was centrifuged at 50 g (600 rpm), the supernatant discarded and resuspended in Williams' medium E, complete (WEC). Centrifugation and resuspension of the cells was performed a second time to remove as much debris as possible.

A viable cell count was performed after diluting an aliquot of the cells with an equal volume of trypan blue solution. The viable cell yield was also calculated.

Test 1:

Total cell yield: 56 x 10^6 cells/mL

Cell viability: 64%

Test 2:

Total cell yield: 124 x 10^6 cells/mL

Cell viability: 67%

The isolated cells were suspended in WEC at a density of approximately 0.2 x 10^6 cells per mL. This cell suspension was dispensed in 2 mL aliquots into multi-well tissue culture plates, each well containing a coverslip. The cultures were incubated at 37°C in a humid atmosphere containing 5% carbon dioxide for a minimum of 90 minutes to allow hepatocytes to attach to the coverslips. After this attachment period the supernatant medium was removed and the cells were gently rinsed with one wash of Williams' medium E, incomplete (WEI).

The medium was then replaced with WEI containing high specific activity (methyl-^{3}H) thymidine (Amersham Biosciences UK Ltd), batch number 233 (Test 1) and 234 (Test 2); specific activity 91 and 86 Ci/mmol, respectively.

Culture treatment: Facin was dosed at twelve half-log_{10} concentrations up to a maximum final concentration of 5 mM in Test 1 and 0.5 mM in Test 2. Each 2 mL culture was treated with a 20 µL aliquot of the vehicle (DMSO), test substance solution or the positive control solution. Six cultures were treated at each concentration except that twenty four cultures were treated with the vehicle control.

The cultures were returned to the incubator for a treatment period of 18 hours. After this labelling period, the medium was removed and replaced by WEI containing 250 µM cold (unlabelled) thymidine (TdR). The cultures were then incubated for a 'chase' period of approximately 24 hours. This additional culture period helps to wash out excess radiolabel and improves cell morphology thus facilitating subsequent grain count analysis of autoradiographs.

Cell harvest: After the 24 hour cold chase with thymidine, coverslips with attached cells were removed from the culture medium, washed in Hanks' balanced salts solution (three washes each of 5 minutes), fixed in 2.5% v/v acetic acid in ethanol (2 washes each of 5 minutes) and washed twice in purified water. The coverslips were allowed to air dry and were mounted on glass microscope slides, with the cell layer uppermost, using DPX mountant. The mountant was allowed to harden.

Autoradiography: Autoradiographs were prepared (***Rogers 1973***) from three cultures for each test substance concentration and positive control and from 12 vehicle control cultures.

After the exposure period (11 days for Test 1 and 8 days for test 2), slides were developed using Kodak D19 developer. The cells were stained using Meyers Haemalum and allowed to air dry.

Examination of slides: The slides were randomised, encoded and grain count analysis performed using a Leitz Laborlux microscope connected to a dedicated Sorcerer image analysis system via a solid state video camera. Prior to grain count analysis the autoradiographs were examined for signs of test substance-induced toxicity (eg pyknosis, reduced levels of radiolabelling). Slides were examined using a 60x oil-immersion objective.

The image analyser was used in the area count mode. Fifty hepatocytes over several widely separated randomly chosen fields of view from twelve vehicle control cultures were analysed. In addition, fifty hepatocytes from each of two cultures at the seven highest non-toxic test substance concentrations and the lowest positive control concentration at which a positive effect was identified were analysed.

Only results from hepatocytes not in S-phase with a normal morphology (i.e. not pyknotic or lysed) without staining artefacts or debris were recorded. For each cell the number of silver grains overlying the nucleus was estimated using the image analysis system, then the number of silver grains in an equivalent and most heavily-grained, adjacent area of cytoplasm was estimated. The cytoplasmic grain count was subtracted from the gross nuclear grain count to give the net nuclear grain count.

Data analysis: Statistical evaluation of both net and gross nuclear grain counts was performed using classical one-way analysis of variance (Snedecor and Cochran 1967) followed by a Student's t-test with an appropriate transformation of values if indicated by excessive variance. Critical probability levels are based on a one-sided distribution.

A positive response is normally indicated by a substantial and reproducible dose-related statistically significant increase in the net nuclear grain count which is accompanied by a substantial increase in the gross nuclear grain count over concurrent solvent control values. Normally the effect would be dose-related with the highest response being observed at concentrations just below the toxic level.

A negative response is indicated by a mean net nuclear grain count which is not significantly greater than the concurrent control.

An equivocal response is obtained when the results do not meet the criteria specified for a positive or a negative response

Results and Discussion

Test 1: Cultures were treated with Facin at twelve half-login concentrations to give final concentrations ranging from 0.0000158 mM to 5 mM. Assessment by microscopic examination prior to harvesting showed that treatment levels of 0.158 mM and above, showed few healthy cells and many dead cells, indicating toxicity.

Seven dose levels selected for scoring were 0.05, 0.0158, 0.005, 0.00158, 0.0005, 0.000158 and 0.00005 mM. The positive control cultures dosed with 2-AAF at the lowest concentration (1 µg/mL) were selected for scoring.

Table IIA 5.4.3-1: Summary of results – Test 1 (100 cells scored from 2 slides)

Treatment	Culture Number	Final concentration (mM)	Mean nuclear count±SD	Mean cytoplasmic count±SD	Mean net grain count±SD
Vehicle control	1	-	12.26±7.00	18.62±9.85	-6.36±6.99
	2	-	13.39±7.58	21.24±10.02	-7.85±7.05
	3	-	13.64±8.88	19.17±11.34	-5.53±6.60
	4	-	11.74±7.22	17.06±8.12	-5.32±5.48
	5	-	17.49±9.09	23.76±11.45	-6.27±7.65
	6	-	10.96±6.61	16.70±9.12	-5.74±6.38
Facin	7	0.00005	12.85±7.19	20.75±9.34	-7.90±7.07
	8	0.000158	10.48±5.69	17.05±7.93	-6.57±5.18
	9	0.0005	10.96±6.86	17.84±8.91	-6.88±6.22
	10	0.00158	11.23±7.13	18.91±9.65	-7.38±7.19
	11	0.005	12.70±7.37	22.52±12.22	-9.82±8.13
	12	0.0158	23.38±10.34	24.86±10.20	-1.48±7.55
	13	0.05	16.56±10.85	24.66±14.03	-8.10±8.05
2-AAF	14	1 (µg/mL)	54.11±27.51	17.79±9.65	36.32±22.18

Test 2: On the basis of data from Test 1, final concentrations of Facin chosen for Test 2 ranged from 0.00000158 mM to 0.5 mM. Assessment by microscopic examination prior to harvesting showed that cultures treated with Facin at 0.5 mM had few live cells indicating toxicity.

Seven dose levels selected for scoring were 0.158, 0.05, 0.0158, 0.005, 0.00158, 0.0005, and 0.000158 mM. The positive control cultures dosed with 2-AAF at the lowest concentration (1 µg/mL) were selected for scoring.

Table IIA 5.4.3-2: Summary of results – Test 2 (100 cells scored from 2 slides)

Treatment	Culture Number	Final concentration (mM)	Mean nuclear count±SD	Mean cytoplasmic count±SD	Mean net grain count±SD
Vehicle control	15	-	12.65±6.34	22.34±10.12	-9.69±9.69
	16	-	12.91±6.70	21.89±10.49	-8.98±7.40
	17	-	18.94±9.35	31.19±14.17	-12.25±11.04
	18	-	13.57±8.01	22.10±12.91	-8.53±9.61
	19	-	11.51±7.97	21.07±11.92	-9.56±8.82
	20	-	9.06±6.82	15.97±10.46	-6.91±7.64
Facin	21	0.000158	8.36±3.45	17.29±6.94	-8.93±5.96
	22	0.0005	9.96±5.18	19.50±9.21	-9.54±7.96
	23	0.00158	11.38±5.88	22.28±9.90	-10.90±7.57
	24	0.005	15.99±8.24	28.87±12.92	-12.96±7.72
	25	0.0158	12.36±5.45	23.24±9.73	-10.88±8.39
	26	0.05	17.25±7.50	27.40±13.39	-10.15±9.96
	27	0.158	14.05±7.73	17.94±10.95	-3.89±7.60
2-AAF	28	1 (µg/mL)	33.81±17.00	13.92±8.22	19.89±14.12

No statistically significant increases in the net nuclear grain count, cytoplasmic grain count or gross nuclear grain count were obtained at any dose level of Facin in either test.

Cultures treated with the positive control agent (2-AAF) showed a statistically significant increase in the net nuclear grain count which was accompanied by an increase in the gross nuclear grain count.

Conclusion

It is concluded that Facin (QRD 406) has not shown any evidence of causing unscheduled DNA synthesis in this *in vitro* test system.

References:

Rogers, A.W. (1973) Techniques In Autoradiography. Elsevier, Amsterdam.

Snedecor, G.W. and Cochran, W.G. (1967) Statistical Methods, 6th ed., Iowa State University Press.

([redacted] L, 2004)

IIA 5.4.4 *In vivo* genotoxicity testing (somatic cells) - Metaphase analysis in rodent bone marrow, or micronucleus test in rodents

QRD 460 has not been tested in this assay, however other genotoxicity screening has been conducted and summarised above and published data exist for the components. These are discussed in Section IIA 5.4 above.

IIA 5.4.5 *In vivo* genotoxicity testing (somatic cells) - Unscheduled DNA synthesis or a mouse spot test

QRD 460 has not been tested in this assay, however other genotoxicity screening has been conducted and summarised above and published data exist for the components. These are discussed in Section IIA 5.4 above.

IIA 5.4.6 *In vivo* studies in germ cells

QRD 460 has not been tested in this assay, however other genotoxicity screening has been conducted and summarised above and published data exist for the components. These are discussed in Section IIA 5.4 above.

IIA 5.5 Long term toxicity and carcinogenicity

No long term or carcinogenicity data exist for QRD 460. However, AgraQuest Inc. believes sufficient data and information are available from other sources to address the repeat toxicity of QRD 460. These arguments are presented in section IIA 5.3. The waiver justifications support the contention that the use of products containing QRD 460 will not result in repeated human exposure to QRD 460 by the oral, inhalation or dermal routes. Furthermore an evaluation of the three terpene components by Structure Activity Relationships (SAR) and established genotoxicity assays is sufficient to allow a conclusion of no significant genotoxic activity. There is therefore no reason to anticipate any carcinogenicity due to a genotoxic action.

The meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) was reported by WHO in 2005[51]. The components of QRD 460, α-terpinene, p-cymene, and d-limonene were all discussed as established flavouring agents used in food. Many flavouring agents occur naturally in foods (coffee, alcoholic beverages, baked and fried potato, heated beans, tea, bread, cheese) and the substances with highest natural occurrence are d-limonene and p-cymene. As previously summarised, the per capita daily consumption of the three main components as <u>food additives</u> in the US and Europe, respectively, are: d-limonene, 12.76 mg and 39.307 mg; p-cymene, 0.472 mg and 1.085 mg; α-terpinene, 0.093 mg and 0.032 mg. The committee considered use of α-terpinene, p-cymene, and d-limonene as flavouring agents in food would not present a safety concern at these estimated current intake values (less than the human intake threshold of 1800 µg/kg/person per day for all except d-limonene). Furthermore the committee concluded an ADI for d-limonene is "not specified" for on the basis of short and long term toxicity studies in rats and mice and developmental toxicity in mice, rats and rabbits. WHO reported d-limonene was tested at dose levels from 250-2800 mg/kg per day and it was concluded that it would pose no safety concerns at the estimated current intakes of 660 µg/kg bw per day in Europe. The establishment of an acceptable daily intake expressed in numerical form was not deemed necessary.

Literature sources: The International Programme on Chemical Safety[52] reviewed d-limonene in 1998 and considered it to be a chemical with fairly low toxicity. Following oral or intraperitoneal administration, the target organ in animals is the liver (except male rats). No inhalation studies were identified at the time of the review. Exposure to limonene affects the activity of different liver enzymes, liver weight, cholesterol levels and bile flow; changes have been reported in mice, rats, and dogs. In male rats, d-limonene exposure causes damage to the kidneys and renal tumours. However the male rat specific protein, alpha2µ-globulin, is considered to play a role in the development of neoplastic as well as non-neoplastic kidney lesions, and therefore these effects are considered not to be relevant for human risk assessment.

Dogs orally dosed with d-limonene at 0.4 - 3.6 ml/kg bw/day (1000 mg/kg bw bitches and 3024 mg/kg bw dogs) for 6 months resulted in slight bodyweight loss due to vomiting in some animals (Tsuji *et al.* 1975[53]). Slightly increased total and relative liver weights and an 35% increase in alkaline phosphatase and cholesterol in serum (but no histopathological changes) were observed in dogs following oral administration of d-limonene at 1.2 ml/kg bw/day for 6 months (equivalent to 1000 mg/kg bw/day) (Webb *et al.* 1990[54]).

In 1990, the National Toxicology Programme[55] tested d-limonene in a 2-year study; groups of 50 male F344/N rats received the material orally at 0, 75, or 150 mg/kg bw day whilst females received 0, 300, or 600 mg/kg bw/day;

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[51] WHO (2005), Evaluation of Certain Food Additives. WHO Technical Report Series No. 928. 63rd Report of the Joint FAO/WHO Expert Committee on Food Additives.

[52] IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene

[53] Tsuji M, Fujisaki Y, Arikawa Y, Masuda S, Tanaka T, Sato K, Noda K, Ide H, Kikuchi M (1975) Studies on d-limonene, as gallstone solubilizer (IV): Chronic toxicity in dogs. (Japan) Oyo Yakuri, 9:775-808. (Cited in IARC Monographs (1995). D-Limonene Volume 73).

[54] Webb DR, Kanerva LL, Hysell DK, Alden CL, Lehman-McKeeman LD (1990). Assessment of the subchronic oral toxicity of *d*-limonene in dogs. Food and chemical toxicology, 28:669-675. (Cited in IARC Monographs (1995). D-Limonene Volume 73).

[55] NTP (1990). Toxicology and carcinogenesis studies of d-limonene in F344/N rats and B6C3F1 mice. Springfield, VA, US Department of Health and Human Services, National Institutes of Health, National Toxicology Program (NTP Technical Report No. 347). (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

B6C3F$_1$ male mice received 0, 250, or 500 mg/kg bw/day and females received 0, 500, or 1000 mg/kg bw/day. Whilst slightly lower body weights were seen in high dose male rats and female mice, there were no other clinical signs. Survival in female rats was reduced after 39 weeks. There was no evidence of carcinogenicity in female rats or in male and female mice at any dose. There was clear evidence of carcinogenic activity in male rats, based upon a dose-related increase in the incidence of hyperplasia and adenoma/adenocarcinoma in renal tubular cells. The carcinogenic response in the kidney of male rats has been linked to a unique renal perturbation involving alpha2μ-globulin and therefore these effects are considered not to be relevant for human risk assessment.

d-Limonene was given orally at 0.5 to 12 g/m^2 body surface area (BSA) to patients with advanced cancer in a Phase 1 clinical trial. The maximum tolerated dose was 8 g/m^2 BSA. Whilst dose related gastrointestinal side effects such as nausea and diarrhoea were observed, the authors reported that d-limonene had low toxicity following single and repeated dosing for up to 1 year (Vigushin et al 1998[56]).

The International Agency for Research on Cancer[57] considered there is inadequate evidence in humans for the carcinogenicity of d-limonene but there is sufficient evidence in experimental animals for carcinogenicity. However the renal tubular tumours in male rats was concluded to be produced by a non-DNA-reactive mechanism, through an alpha2μ-globulin associated response, therefore the mechanism by which d-limonene increases the incidence of renal tumours is not relevant to humans. IARC classified d-limonene in Group 3 (not classifiable as to its carcinogenicity to humans).

In summary, no reasonable likelihood for repeated human exposure to QRD 460 is foreseen by the oral route, inhalation or dermal rotes. With limited occupational exposure and no exposure to residual product post-application, there is insignificant potential for repeated exposure to the terpene components in QRD 460. An evaluation of the three terpene components of QRD 460 by SAR and established genotoxicity assays is sufficient to allow a conclusion of no significant genotoxic activity. There is therefore no reason to anticipate any carcinogenicity due to a genotoxic action. Furthermore, the data on the terpene components generally indicate low toxicity and there are data for d-limonene indicating that after repeated administration to rats and mice, the only tumours noted were in the male rat kidney and these have been generally accepted as due to a non-genotoxic mechanism, namely through the accumulation of alpha 2U-globulin. Further use of animals in conducting long term studies on these chemicals is considered unnecessary.

IIA 5.5.1 Long-term (2 years) oral toxicity in the rat (can be a combined long-term and carcinogenicity study)

No long term or carcinogenicity data exist for QRD 460, however sufficient data and information are available from other sources and are discussed in Section IIA 5.5 above.

IIA 5.5.2 Carcinogenicity study in the rat (can be a combined long-term and carcinogenicity study)

No long term or carcinogenicity data exist for QRD 460, however sufficient data and information are available from other sources and are discussed in Section IIA 5.5 above.

IIA 5.5.3 Carcinogenicity study in the mouse

No long term or carcinogenicity data exist for QRD 460, however sufficient data and information are available from other sources and are discussed in Section IIA 5.5 above.

IIA 5.5.4 Mechanism of action and supporting data

No data exist for QRD 460, however sufficient data and information are available from other sources and are discussed in Section IIA 5.5 above.

[56] Vigushin DM, Poon GP, Boddy A et al (1998). Phase 1 and pharmacokinetic study of d-limonene in patients with advanced cancer. Cancer Chemother Pharmacol 42, 111-117.

[57] IARC Monographs (1995). D-Limonene Volume 73.

IIA 5.6 Reproductive toxicity

Prompted by a journal article indicating that α-terpinene had embryofetoxic effects, QRD 420 has been tested in a developmental toxicity study in rats. The maternal and developmental no-observed-adverse-effect level (NOAEL) for QRD 420 reported in this study was 60 mg/kg/day. No embryo/foetal developmental toxicity were seen at any dose level in the absence of maternal toxicity. QRD420 is similar in composition to QRD 460 except that it also contains canola oil (see Document J).

Literature sources Of the QRD components, most published data are available for d-limonene. Whilst no studies were available on the reproductive toxicity of limonene, there is no evidence that limonene has teratogenic or embryotoxic effects in the absence of maternal toxicity[58].

2869 mg/kg bw/day d-limonene was orally administered to rats on days 9-15 of gestation; decreased body weight and deaths was seen in the dams and this was accompanied by delayed ossification, decreased total body and organ weights (thymus, spleen, and ovary) in the offspring (Tsuji *et al.* 1975[59]). Similar effects were seen in mice orally administered with 2869 mg/kg bw/day d-limonene on days 7-12 of gestation; reduced growth was seen in the dams and a significantly increased incidence of skeletal anomalies and delayed ossification in the offspring (Kodama et al., 1977a[60]). However no dose related trends were seen in rabbits administered the material at 250 - 1000 mg/kg bw/day) on days 6-18 of gestation; at 1000 mg/kg there was mortality and reduced weight gain among the dams and at 500 mg/kg bw/day, maternal bodyweights were decreased (Kodama *et al.* 1977b[61]).

In humans limonene has been detected, but not quantified, in breast milk of non-occupationally exposed mothers (Pellizzari *et al.* 1982[62]).

The embryotoxicity of α- terpinene is reported by Araujo *et al.* (1996)[63]. Rats were dosed orally at 0, 30, 60, 125 and 250 mg/kg bw/day in corn oil from day 6-15 of gestation. On day 21 of pregnancy caesarean sections were done and the number of implantation sites, living/dead foetuses, resorptions and corpora lutea were recorded. Foetuses were weighed and examined for externally visible malformations and one-third of the foetuses of each litter were evaluated for visceral anomalies by a micro sectioning technique. The authors reported a maternal NOAEL, based on reduced weight gain at the two highest dose levels, of 60 mg/kg. No increase in the ratio of resorptions/implantations was observed over the dose range tested. The developmental NOAEL was reported to be 30 mg/kg based developmental effects which included signs of delayed ossification (poorly ossified and not ossified bones as well as irregular spongy bones) and a higher incidence of minor skeletal malformations at 60 mg/kg.

As both QRD 460 and structurally similar QRD 420 contain α- terpinene, it was these data that prompted testing of QRD 420 in a rat developmental toxicity study. Full details of this study are reported under point IIA 5.6.10 below. In addition, a separate review has been conducted to re-evaluate the results of each rat developmental toxicity study ([redacted] 2011). This review has led to a revision of the conclusion on the NOAELs as stated for the individual studies. The revised outcomes of the two studies are compared in the table below.

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[58] IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene

[59] Tsuji M, Fujisaki Y, Okubo A, Arikawa Y, Noda K, Ide H, Ikeda T (1975) Studies on d-limonene, as gallstone solubilizer (V): Effects on development of rat fetuses and offspring. (Japan) Oyo Yakuri, 10:179-186. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

[60] Kodama R, Okubo A, Araki E, Noda K, Ide H, Ikeda T (1977a) Studies on d-limonene as a gallstone solubilizer (VII). Effects on development of mouse fetuses and offspring. (Japan) Oyo Yakuri, 13:863-873. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

[61] Kodama R, Okubo A, Sato K, Araki E, Noda K, Ide H, Ikeda T (1977b). Studies on d-limonene as a gallstone solubilizer (IX). Effects on development of rabbit fetuses and offspring. (Japan) Oyo Yakuri, 13:885-898. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

[62] Pellizzari ED, Hartwell TD, Harris BSH III, Wadell RD, Whitaker DA, Erickson MD (1982) Purgeable organic compounds in mother's milk. Bulletin of environmental contamination and toxicology, 28:322-328. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

[63] Araujo IB, Souza CAM, De-Carvalho RR, Kuriyama SN, Rodrigues, RP, Vollmer RS, Alves EN and Paumgartten FJR (1996). Study of the embryofoetotoxicity of α-terpinene in the rat. Food and Chemical Toxicology Volume 34, Issue 5, May 1996, Pages 477-482

Table IIA 5.6-1: QRD 420 and α-Terpinene: Comparison of rat developmental toxicity studies

Endpoint	α-Terpinene (89% pure)	QRD 420 (containing 38.8 % α-terpinene)
Reference	Araujo IB et al., 1996	[redacted] 2009
Dose levels (mg/kg/day) Dosing period Terminal examinations	0, 30, 60, 125 and 250 gestation days 6-15 inclusive gestation day 21	0, 60, 120 and 240 gestation days 6-19 inclusive gestation day 20
Implantation Loss	Only 56% of mated females given 250 mg/kg/day were pregnant at termination due to whole-litter losses at the time of implantation.	No effect at highest dose of 240 mg/kg/day on implantation.
Maternal Body Weight	Severe effect (weight loss) following the start of treatment with 250 mg/kg/day. Reduced weight gain with 125 mg/kg/day; a more severe effect than that of 120 mg QRD 420/kg/day. NOAEL 60 mg/kg/day	Reduced maternal weight gain due to 240 mg/kg/day and to a lesser extent to 120 mg/kg/day NOAEL 60 mg/kg/day
Foetal Body Weight	Lower foetal body weight with 250 mg/kg/day - lower by 14.9 % following 10 daily doses as measured on day 21. No effect of 125 mg/kg/day.	Lower foetal body weight with 240 mg/kg/day - lower by 8.6% following 14 daily doses as measured on day 20. No effect of 120 mg/kg/day.
Foetal Anomalies	No teratogenicity	No teratogenicity
Foetal Skeletal Observations	Altered ossification of a small number of bones at 125 and 250 mg/kg/day.	Altered ossification of a small number of bones at 240 mg/kg/day.
NOAEL maternal toxicity	60 mg/kg/day	60 mg/kg/day
NOAEL developmental toxicity	125 mg/kg/day *(report concludes 30 mg/kg/day)*	120 mg/kg/day *(report concludes 60 mg/kg/day)*

The magnitude of the effects of treatment on the pregnant rat is greater with α-terpinene than with QRD 420. This is to be expected given that QRD 420 contains only 38.8 % α-terpinene. However, the types of effect seen are generally similar.

Both substances induce maternal toxicity causing a reduction in body weight gain particularly in the period shortly following the onset of treatment, around the time of major organogenesis. The effects occur with similar nominal dose levels (125/120 and 250/240 mg/kg/day) although the severity of the effect is much greater with α-terpinene than with QRD 420. In addition, the onset of treatment with highest dose level of α-terpinene, 250 mg/kg/day, caused whole litter loss in 44% of the mated females. The NOAEL for maternal toxicity was 60 mg/kg/day for both substances.

At the highest dose levels tested, both substances caused a reduction in mean foetal weight, probably as a consequence of the effect on maternal body weight, and hence the greater effect of α-terpinene. Ten daily doses of α-terpinene on days 6-15 of gestation resulted in a mean foetal weight 14.9 % lower than in controls, on day 21 i.e. following 5 treatment-free days. In comparison, 14 daily doses of QRD 420 on days 6-19 of gestation resulted in a mean foetal weight 8.6 % lower than in controls on day 20 i.e. on the day following the last dose.

There was no evidence of teratogenicity or abnormal foetal development due to treatment with either substance.

Altered ossification of a small number of bones of the foetal skeleton was observed for both substances. Only a small number of ossification centres were affected yet those altered following maternal treatment with QRD 420 were also those altered by maternal treatment with α-terpinene. It is arguable whether these minor temporal alterations in only a few of the many skeletal components should be regarded as evidence of developmental toxicity. At the highest dose levels tested, 250 mg α-terpinene/kg/day and 240 mg QRD 420/kg/day, it is the reduction in mean foetal weight that defines the developmental toxicity. With 125 mg α-terpinene/kg/day altered ossification is confined to only four areas of the skeleton and with 120 mg QRD 420/kg/day and also 60 mg α-terpinene/kg/day only one area of the

skeleton is affected. These changes are considered too minor to be of toxicological significance and in themselves are considered not to provide evidence of developmental toxicity.

The review concludes that the NOAEL for maternal toxicity in rat developmental toxicity studies was 60 mg/kg/day for α-terpinene and for QRD 420, containing 38.8 % α-terpinene. Developmental toxicity was seen only in the presence of maternal toxicity and a reduction in foetal weight. The NOAEL for developmental toxicity was 125 mg/kg/day for α-terpinene and 120 mg/kg/day for QRD 420, containing 38.8 % α-terpinene. These conclusions differ from those of the authors for each study and reflect different interpretations regarding the toxicological significance of the reported changes in foetal ossification as described in the previous paragraph.

There was no evidence of teratogenicity or abnormal foetal development in the rat, due to maternal treatment with α-terpinene or QRD 420 containing 38.8 % α-terpinene during the period of organogenesis, at dose levels that induced maternal toxicity. Minor, transient alterations in the ossification of a few areas of the foetal skeleton were seen only in the presence of maternal toxicity and reduced foetal weight.

There are no further data in the literature indicating adverse effects on reproduction despite their extensive use as food additives.

IIA 5.6.1 Two generation reproductive toxicity in the rat

Not required, see section IIA 5.6 above.

IIA 5.6.2 Separate male and female studies

Not required, see section IIA 5.6 above.

IIA 5.6.3 Three segment designs

Not required, see section IIA 5.6 above.

IIA 5.6.4 Dominant lethal assay for male fertility

Not required, see section IIA 5.6 above.

IIA 5.6.5 Cross-matings of treated males with untreated females and *vice versa*

Not required, see section IIA 5.6 above.

IIA 5.6.6 Effect on spermatogenesis

Not required, see section IIA 5.6 above.

IIA 5.6.7 Effects on oogenesis

Not required, see section IIA 5.6 above.

IIA 5.6.8 Sperm motility, mobility and morphology

Not required, see section IIA 5.6 above.

IIA 5.6.9 Investigation of hormonal activity

All available information supports the conclusion that QRD 460 is not comprised of estrogenic, anti-estrogenic, androgenic or anti-androgenic components. The terpene components are naturally occurring materials that are present in fruit and vegetables. To date, there is no evidence in the published literature to suggest that natural exposure to components of QRD 460 affect the immune system, function in a manner similar to any known hormone, or that they act as endocrine disruptors. Moreover, the use of QRD 460 is not expected to result in any significant exposures,

effectively obviating any opportunity for negative effects on humans or the environment. Presently, based on the lack of exposure and the low toxicity profiles of the natural and synthetic extracts, no adverse effects to the endocrine or immune systems are known or expected.

IIA 5.6.10 Teratogenicity test by the oral route in the rat

Report:	IIA 5.6.10/01 [redacted] W, 2009. A developmental toxicity study of orally administered QRD 420 in rats. [redacted], IL 60616-3799. Laboratory Report No. 2212-001, 12 June 2009, Unpublished

Guidelines

Prenatal Developmental Study (rat) OECD 414: OPPTS 870.3700

GLP: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

Executive Summary

In a developmental study, QRD 420 was administered orally, by gavage, to time-mated primiparous female COBS:CD, IGS rats during the major organogenesis period. Four groups, each of 25 rats, were tested at dose levels of 0, 60, 120 or 240 mg/kg bw/day, on gestation days 6-19 inclusive.

Dam body weights, weight gain and food consumption were measured throughout the study. The rats were sacrificed on Gestation Day 20. Caesarean sections were performed on all surviving, gravid rats. For each animal, corpora lutea on each ovary were counted and the uterus was removed, weighed and examined for implantation sites and tissue resorptions. Foetuses were sexed, weighed and subjected to gross external, visceral, cephalic and skeletal examinations that allowed for assessment of developmental abnormalities.

Statistically significant decreases in maternal body weight, body weight gain and food consumption were seen at the 240 mg/kg dose level, and statistically significantly decreased total body weight gain and Gestation Day 20 food consumption were seen at the 120 and 240 mg/kg dose levels. Adjusted body weight (uterus weight subtracted from final body weight) was statistically significantly decreased at the 240 mg/kg dose level, and adjusted total weight gain (uterus weight subtracted from total gain) was statistically significantly decreased at the 120 and 240 mg/kg dose levels.

No statistically significant differences in the mean number of corpora lutea, total implantation sites, percent pre- or post-implantation loss, live or dead foetuses per litter, or total resorptions per dam were observed in the test substance-treated groups compared to the vehicle control group. No increase in the incidence of any gross external, visceral or cephalic abnormalities was observed at any dose level.

Signs of foetal toxicity consisted of a statistically significant decrease in body weight at the 240 mg/kg dose level, and skeletal variations at the 120 and 240 mg/kg dose levels. Skeletal variations consisted primarily of signs of delayed ossification and irregularly shaped squamosal bones in the skull. The decrease in foetal body weight and the associated skeletal variations were considered secondary to the maternal toxicity seen at the 120 and 240 mg/kg dose levels.

In conclusion the maternal and developmental no-observed-adverse-effect level (NOAEL) for QRD 420 was 60 mg/kg/day. However, no embryo/foetal developmental toxicity were seen at any dose level in the absence of maternal toxicity.

Materials:

Test Material:	QRD 420
Description:	Pale yellow liquid
Lot/Batch number:	08-137GJ-1
Purity:	100%
CAS#:	Not reported
Stability of test compound:	Not reported

Vehicle and/or positive control: Food-grade canola oil

Test Animals:

Species	Rat
Strain	COBS:CD, IGS
Age/weight at dosing	Approximately 13-14 weeks/ weight on day 1 of gestation 190-268 g
Source	[redacted]
Housing	Individually housed in polycarbonate "shoe box" cages
Acclimatisation period	1-4 days
Diet	Certified Rodent Diet #5002 [redacted] *ad libitum*
Water	Municipal tap water *ad libitum*
Environmental conditions	Temperature: 21-23°C Humidity: 31-62% Air changes: minimum of 10 per hour Photoperiod: 12 hours light / 12 hours dark

Study Design and Methods:

In-life dates: Start: 16 September 2008 End: 03 October 2008

Mating procedure: Time-mated pregnant rats were used.

Animal assignment: Animals were randomly assigned to test groups, using an in-house developed computerized randomization procedure based on body weights, as shown in the following table:

Table IIA 5.6.10-1: Animal numbers and treatment groups

Dose level of QRD 420 (mg/kg/day)			
0 (control)	**60**	**120**	**240**
25	25	25	25

Dose selection rationale: Not reported.

Diet/dosage preparation and analysis: Dosing formulations of the test substance were prepared weekly at concentrations of 12, 24 and 48 mg/mL (for the 60, 120 and 240 mg/kg/day dose levels respectively. The dosing volume was 5 mL/kg body weight and the vehicle was food-grade canola oil. Each formulation was prepared independently by weighing the appropriate aliquot of the test substance into a labelled, calibrated flask. Vehicle was added to the flask and the formulation was mixed until homogeneous. The formulation was brought to final volume with additional vehicle, mixed, and then transferred to a labelled dosing jar that was stored at room temperature.

Achieved concentrations and homogeneity and stability (over 8 days at room temperature) were determined on the initial batch of dosing formulations.

Concentration analysis results: Analysis of the dose formulations determined that all formulations tested were within 10% of the target test substance concentration. For the dose formulations with target concentrations of 12, 24 and 48 mg/mL, the percent relative standard deviations (%RSD) were 2.0, 0.15 and 4.2%, respectively, and the overall accuracies (% of target) were 93, 91 and 92%, respectively.

Homogeneity results: QRD 420 dose formulations were homogeneous, with %RSDs of 2.0 and 4.2 for the 12 and 48 mg/mL dose formulations, respectively.

Stability results: A one-week stability study of the 12 and 48 mg/mL QRD 420 dose formulations at room temperature indicate that QRD 420 dose formulations can be stored at room temperature for at least eight days without degradation.

Dosage administration: All doses were administered once daily by oral intubation (gavage, on gestation days 6 to 19, at a constant dosing volume of 5 mL/kg body weight/day. Dosing was based on individual bodyweight at the time of dosing.

Observations:

Maternal observations: Animals were examined for signs of toxicity and mortality twice daily, at least 4 hours apart. Complete physical examinations were performed on the day of randomisation. Subsequently they were examined for signs of pharmacological or toxic effects approximately 1-2 hours after dosing on gestation days 6-19.

Initial (Day 0) body weights for all groups were provided by the animal supplier. Thereafter, each rat was weighed on Gestation Days 6, 9, 12, 15, and at study termination (Gestation Day 20).

Food consumption was monitored on Days 6, 9, 12, 15, and at study termination (Gestation Day 20).

Any females that died or were killed *in extremis* during the study were examined macroscopically. Any abnormalities or lesions, the number of corpora lutea per ovary and the number of foetuses were recorded. Uteri that appeared non-gravid were further examined by ammonium sulphide staining to confirm the non pregnant status.

All surviving rats were killed on gestation day 20 and given a caesarean section. Tissue masses and suspect lesions, if present, were collected and prepared for histopathological examination. The uterus was removed from the dam, trimmed of excess adherent fat and weighed, with the ovaries, prior to removal of the foetuses from the uterine horns. After weighing, the corpora lutea were counted and recorded for the left and right ovaries. Uteri that appeared non-gravid were further examined, by ammonium sulfide staining, to confirm the non-pregnant status. Each uterine born was inspected for implantations and the contents were recorded and classified as:

- early resorption (placenta only or unrecognizable foetal tissue)

- late resorption (placenta with an autolysed recognizable foetus)

- dead foetus (foetus with no signs of autolysis)

- live foetus (foetus pink in colour and responds to touch)

Foetal observations: The foetuses were removed, counted, weighed and given an external morphological examination. All foetal abnormalities were recorded. Observations (e.g. normal, within normal limits) and foetal variations or malformations were documented for each foetus. Sex was determined by ano-genital distance. Approximately one-half of the foetuses from each litter received a visceral and cephalic examination. Foetuses not chosen for visceral/cephalic examination were processed for skeletal evaluation. Foetuses were randomly chosen for either visceral/cephalic or skeletal evaluation; however, dead foetuses were designated for skeletal examination. Observations (normal or abnormal findings) were documented for each foetus.

Cephalic Examination: Decapitated foetal heads from approximately one-half of each litter were fixed in Bouin's solution for a minimum of one week prior to an examination using a modified Wilson's Razor Blade Technique. A stereomicroscope was used, as needed, to examine the cephalic structures and organs. All abnormalities were recorded and classified as variations or malformations. Foetal heads were preserved in 70% ethanol.

Visceral Examination: Visceral examinations were performed on approximately one-half of the foetuses from each litter using a modified Staples Technique. The soft tissues (excluding the head) of each foetus were examined using a stereomicroscope. Foetuses were sexed prior to visceral examination and again internally for verification purposes during the visceral examination. Adverse or abnormal findings were classified as variations or malformations.

Skeletal Examination: Approximately one-half of the foetuses from each litter were processed for skeletal examination. Foetuses were eviscerated, skinned, fixed in 95% ethanol, stained with Alcian Blue, and then stained with Alizarin Red-S/potassium hydroxide solution and cleared. A stereomicroscope was used, as needed, for counting and examining the cartilage and skeletal formations. All skeletal abnormalities were recorded and classified as variations or malformations. Foetal skeletons were preserved in glycerin.

Statistical analyses: Means and standard deviations were calculated for all applicable parameters, including litter body weights. The dam was considered the unit of statistical evaluation. Maternal body weights, body weight gain and food consumption data were analyzed by analysis of variance (ANOVA) using LABCAT. Dam uterus weights and adjusted body weights/gains as well as foetal weight data were analyzed by ANOVA using SYSTAT. In the presence of a significant effect, post hoc comparisons were performed using Dunnett's test. Minimum significance levels of $p \leq 5\ 0.05$ (SYSTAT) or $p < 0.05$ (LABCAT) were used in the comparisons.

The incidence, or the means and standard deviations of maternal and foetal observations were calculated. Calculations and statistical analysis of caesarean section and foetal parameters were performed using the litter (number gravid on Gestation Day 20) or viable litter (if the parameter required an intact foetus) as the unit of analysis. The number of corpora lutea, implantations, viable (live) and nonviable (dead) foetuses, and early and late resorptions and gravid uterine weights were calculated as the total number for each group divided by the number of litters evaluated. Foetal weights by litter and sex were calculated in two steps. First, the individual foetal weights were used to determine the mean foetal weight for each litter. Second, the litter means were used to determine the mean for each group. Pre- and post-implantation loss were calculated by determining the percent loss for each litter followed by group mean calculations. The mean number of corpora lutea, total implantation sites, viable (live) foetuses, resorptions, percentage pre- and post-implantation loss, and foetal body weights, by litter and by sex, were analyzed by one-way ANOVA. In the presence of a significant effect ($p \leq 0.05$), Dunnett's test was used for pair-wise comparisons to the control group. Caesarean section and foetal analyses were performed using SYSTAT. The incidences of selected skeletal variations (by foetus for individual alterations: supraoccipital, reduced ossification; squamosal; irregular; sternabrae, unossified and incomplete ossification; by foetus and by litter for grouped alterations: supraoccipital and squamosal) were compared using the Chi-Square test with the foetus and/or the litter as the experimental unit. Chi-Square tests were performed using SYSTAT.

Indices: The following indices were calculated from caesarean section records of animals in the study: % pre-implantation loss, % post-implantation loss, % live implants, % non-live implants, % litters with deaths, % viable litters with resorptions, % viable litters with abnormal foetuses, % abnormal/live implants, % non-live and abnormal/total implants.

Results

Maternal toxicity:

Mortality and clinical signs: No deaths occurred during the study. All females survived to scheduled caesarean section on Gestation Day 20. One 240 mg/kg female exhibited redness around the nose fur and red vaginal discharge. All other animals were described as normal throughout the study.

Bodyweight: On Gestation Days 9, 12, 15 and 20, body weights were statistically significantly decreased in 240 mg/kg animals compared to controls. Body weight gains on Gestation Days 9, 12 and 20 and total body weight gain were statistically significantly decreased in 240 mg/kg/day animals compared to controls. Total body weight gain was also statistically significantly decreased in 120 mg/kg/day animals compared to controls.

Table IIA 5.6.10-2: Intergroup comparison of bodyweight (g) – selected days

	Dose level of QRD 420 (mg/kg/day)			
day	**0 (control)**	**60**	**120**	**240**
9	260	263	261	245**
12	279	282	279	258**
15	301	304	297	276**
20	364	363	353	328**

** Statistically significant difference from control group mean, p<0.01

Table IIA 5.6.10-3: Intergroup comparison of bodyweight gain (g)

	Dose level of QRD 420 (mg/kg/day)			
day	**0 (control)**	**60**	**120**	**240**
9	19	18	15	3**
12	19	19	18	13**
15	22	21	19	18
20	63	59	56	52**
total	123	118	108**	86**

** Statistically significant difference from control group mean, p<0.01

Food consumption: On Gestation Days 9, 12, 15 and 20, food consumption was statistically significantly decreased in 240 mg/kg/day animals compared to controls. Food consumption on Gestation Day 20 was also significantly decreased in 120 mg/kg/day animals compared to controls.

Table IIA 5.6.10-4: Intergroup comparison of food consumption (g)

	Dose level of QRD 420 (mg/kg/day)			
day	**0 (control)**	**60**	**120**	**240**
9	45	45	43	30**
12	57	57	53	41**
15	60	61	57	52**
20	108	110	100*	98**

* Statistically significant difference from control group mean, p<0.05

** Statistically significant difference from control group mean, p<0.01

Sacrifice and pathology:

Gross pathology: One 240 mg/kg/day dam had a small spleen, pale liver, and red left ovary, encased in clotted blood.

Uterus weight: Adjusted body weight (uterus weight subtracted from final body weight) was statistically significantly decreased at 240 mg/kg/day), and adjusted total weight gain (uterus weight subtracted from total gain) was statistically significantly decreased at 120 and 240 mg/kg/day compared to controls.

Table IIA 5.6.10-5: Intergroup comparison of uterus weight, adjusted body weight and adjusted body weight gain (g)

	Dose level of QRD 420 (mg/kg/day)			
	0 (control)	**60**	**120**	**240**
Uterus weight	79	72	78	70
Adjusted body weight	286	291	275	259*
Adjusted total body weight gain	44	46	29*	16*

* Statistically significant difference from control group mean, $p<0.05$

** Statistically significant difference from control group mean, $p<0.01$

Caesarean section data: Data are summarised in the table below:

No statistically significant differences in the mean number of corpora lutea, total implantation sites, percent pre- or post-implantation loss, live or dead foetuses per liter, or total resorptions per dam or sex ratio were observed in the test substance-treated groups compared to the vehicle control group.

Table IIA 5.6.10-6: Caesarean section observations for all pregnant females

Observation	Dose level of QRD 420 (mg/kg/day)			
	0 (control)	**60**	**120**	**240**
Initial group size (sperm positive)	25	25	25	25
Animals Pregnant	25	25	23	25
Number of viable litters	25	25	23	24
Number of non-viable litters	0	0	0	1
Nonpregnant	0	0	2	0
Total number of corpora lutea	344	337	331	366
(mean ± SD)	14±2.1	13±2.1	14±2.7	15±2.5
Total number of implants	341	323	318	327
(mean ± SD)	14±2.0	13±2.8	14±2.2	13±2.2
% pre-implantation loss (mean ± SD)	5±9.4	5±10.7	4±5.4	10±9.6
Total number of live implants (foetuses)	322	289	297	297
(mean ± SD)	13±1.9	12±2.8	13±2.6	12±3.4
% post-implantation loss (mean ± SD)	5±8.8	10±13.1	6±11.8	9±20.6
% live implants (out of total implants)	94	89	93	91
Number of deaths (implants)	0	0	0	13
(mean ± SD)	0±0.0	0±0.0	0±0.0	1±2.6
% non-live implants (out of total implants)	6	11	7	9
Litters with deaths	0	0	0	1
% litters with deaths	0	0	0	4
Number of resorptions	19	34	21	17
(mean ± SD)	1±1.4	1±1.7	1±1.7	1±1.0
Litters with resorptions	9	12	8	11
% viable litters with resorptions	36	48	35	44
Number of abnormal foetuses	5	6	6	9
Litters with abnormal foetuses	5	6	5	7

% viable litters with abnormal foetuses	20	24	22	29
% abnormal/live implants	2	2	2	3
% non-live and abnormal/total implants	7	12	9	12
Sex ratio (male:female %)	55:45	52:48	48:52	50:50

Developmental Toxicity:

Foetal body weights: Male, female and combined sexes foetal body weights were statistically significantly decreased in 240 mg/kg/day animals compared to controls.

Table IIA 5.6.10-7: Intergroup comparison of foetal body weight (g)

	Dose level of QRD 420 (mg/kg/day)			
	0 (control)	**60**	**120**	**240**
Male (no litters/group)	4.02±0.209 (25)	4.14±0.247 (24)	4.02±0.294 (23)	3.72±0.425* (25)
Female (no litters/group)	3.84±0.260 (24)	3.84±0.266 (25)	3.79±0.264 (23)	3.51±0.376* (25)
Combined sexes (no litters/group)	3.95±0.220 (25)	3.99±0.268 (25)	3.90±0.265 (23)	3.61±0.380* (25)

* Statistically significant difference from control group mean, $p<0.05$

External examinations: There were no treatment-related incidences of gross external abnormalities (malformations or variations).

Visceral examinations: There were no treatment-related incidences of visceral abnormalities (malformations or variations).

Cephalic examinations: There were no treatment-related incidences of cephalic abnormalities.

Skeletal examinations: Test substance-related skeletal abnormalities were observed in the supraoccipital and squamosal bones of the skull, and in the stemebrae of the pectoral girdle. Using the foetus as the experimental unit, there was a statistically significant increase in the incidence of skeletal abnormalities (all alterations combined) in the supraoccipital and squamosal bones at 240 mg/kg/day, and a statistically significant increase in the incidence of abnormalities in the sternebrae at 120 and 240 mg/kg/day; these changes were not statistically significant using the litter as the experimental unit.

Skeletal abnormalities in the supraoccipital bones consisted of reduced ossification and discontinuity, while irregular shape was the primary abnormality seen in the squamosal bones. In an analysis of individual alterations by foetus, the incidence of irregularly shaped squamosal bones was statistically significantly increased at 240 mg/kg/day. The primary treatment-related abnormality seen in the sternebrae was unossified 5th and/or 6th stemebrae. In an analysis of individual alterations by foetus, the incidence of this abnormality was statistically significantly increased at 120 and 240 mg/kg/day. No other statistically significant increases in any skeletal abnormalities were seen at any dose level. All observed skeletal abnormalities were considered variations not malformations.

Conclusion

In conclusion, the maternal and developmental no-observed-adverse-effect level (NOAEL) for QRD 420 was 60 mg/kg/day. However, no embryo/foetal developmental toxicity were seen at any dose level in the absence of maternal toxicity.

([redacted]W, 2009)

IIA 5.6.11 Teratogenicity test by the oral route in the rabbit

Not required, see section IIA 5.6 above.

IIA 5.7 Neurotoxicity

Sufficient data and information are available from other sources to address the neurotoxicity potential of QRD 460 and hence there is no need to conduct further studies.

Low Toxicity Studies on QRD 460 establish that the active substance is not highly acutely toxic to mammals. The acute oral LD_{50} in rats is 2,000 mg/kg, the acute dermal LD_{50} is >5,050 mg/kg and the acute LC_{50} in rats is > 5.30 mg/L. In the acute oral toxicity study, mortality occurred only at the 2000 mg/kg level. Clinical signs in survivors included diarrhoea, piloerection, polyuria and salivation. Animals that died on test exhibited decreased activity, body tremors and staggered gait, macroscopic findings in these animals revealed gas in the gastrointestinal tract and discoloured content in the large intestine. These clinical signs are indicative of general toxicity rather than a more direct effect of the chemical. No clinical signs of toxicity were seen at the limit doses of the dermal and inhalation toxicity studies.

Literature sources: Again, available only for d-limonene. Oral administration of 3ml to rats and mice resulted in decreased motor activity/lethargy[64]. Similar effects were reported by NTP[65] in mice and rats orally dosed with 1000 mg/kg bw/day (mice) or 1200 mg/kg bw /day (rats). Despite extensive use of/exposure to the components of QRD 460, there are no indications of neurotoxicity in the literature.

None of 8 human subjects reported any discomfort, irritation or symptoms related to central nervous system effects during a 2 hour inhalation exposure to d-limonene at 10, 225 or 450 mg/m^3 (Falk Filipsson *et al.*, 1993[66])

IIA 5.7.1 Acute neurotoxicity – rat

Not relevant (see IIA 5.7 above).

IIA 5.7.2 Delayed neurotoxicity following acute exposure

Not relevant (see IIA 5.7 above).

IIA 5.7.3 28-day delayed neurotoxicity

Not relevant (see IIA 5.7 above).

IIA 5.7.4 Subchronic neurotoxicity – rat –90 day

Not relevant (see IIA 5.7 above).

[64] Tsuji M, Fujisaki Y, Yamachika K, Nakasami K, Fujisaka F, Mito M, Aoki T, Kinoshita S, Okubo A, Watanabe I (1974) Studies on d-limonene, as gallstone solubilizer (I): General pharmacological studies. (Japan) Oyo Yakuri, 8:1439-1459. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

[65] NTP (1990). Toxicology and carcinogenesis studies of d-limonene in F344/N rats and B6C3F$_1$ mice. Springfield, VA, US Department of Health and Human Services, National Institutes of Health, National Toxicology Program (NTP Technical Report No. 347). Cited in Health Canada (2008). Re-evaluation note: Review of the 2004 Re-evauation of Citronella Oil and related active compounds for use as personal insect repellants. REV2008-03

[66] Falk Filipsson A, Löf A, Hagberg M, Wigaeus Hjelm E, Wang Z (1993). d-Limonene exposure to humans by inhalation: Uptake, distribution, elimination, and effects on the pulmonary function. Journal of toxicology and environmental health, 38:77-88. (Reported in NICNAS. Limonene Priority Existing Chemical Assessment Report No 22, May 2002).

IIA 5.7.5 Postnatal development neurotoxicity

Not relevant (see IIA 5.7 above).

IIA 5.8 Toxicity studies on metabolites

Based on the available published data on the terpene components detailing mode of action and breakdown pathways, there are no metabolites of concern.

IIA 5.9 Medical data

IIA 5.9.1 Report on medical surveillance on manufacturing plant personnel

Routine medical surveillance is carried out on all manufacturing plant personnel, and together with routine health and safety records indicate no adverse reactions to the terpene components during manufacture and packaging of QRD 460.

IIA 5.9.2 Report on clinical cases and poisoning incidents

No clinical cases, adverse effects or poisoning incidents reported.

IIA 5.9.3 Observations on exposure of the general population and epidemiological studies

Literature sources: Exposure data were identified for d-limonene. None of 8 human subjects reported any discomfort, irritation or symptoms related to central nervous system effects during a 2 hour inhalation exposure to d-limonene at 10, 225 or 450 mg/m^3 (Falk Filipsson *et al* 1993[67]); however a slight decline (2%) in vital capacity was observed following exposure at the highest concentration.

De Bertoli (1986[68]) measured indoor concentrations of limonene (enantiomer not specified) and reported them to range from 10 to 480 µg/m^3 (mean 140 µg/m^3) whereas Montgomery *et al.,* 1989[69] reported ranges from 1.6 to 78 µg/m^3 (mean 18 µg/m^3) in 17 residences in Ruston, Washington. A mean indoor concentration was measured in Los Angeles, California to be 40 µg/m^3 (Wallace *et al.* 1991[70]). Fellin and Otson, (1993[71]) reported concentrations to be higher in winter when there was reduced ventilation.

In 1994, IPCS reported the intake of limonene from indoor and outdoor air for the general population is estimated to be 10 and 0.1 µg/kg body weight per day, respectively, based on the daily inhalation volume for adults of 22 m^3, a mean body weight for males and females of 64 kg and the assumption that 4 of 24 hours are spent outdoors. The

[67] Falk Filipsson A, Löf A, Hagberg M, Wigaeus Hjelm E, Wang Z (1993). d-Limonene exposure to humans by inhalation: Uptake, distribution, elimination, and effects on the pulmonary function. Journal of toxicology and environmental health, 38:77-88. (Reported in NICNAS. Limonene Priority Existing Chemical Assessment Report No 22, May 2002).

[68] De Bertoli M, Knöppel H, Pecchio E, Peil A, Rogora L, Schauenburg H, Schlitt H, Vissers H (1986) Concentrations of selected organic pollutants in indoor and outdoor air in Northern Italy. Environment international, 12:343-350. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

[69] Montgomery D, Kalman DA (1989) Indoor/outdoor air quality: Reference pollutant concentrations in complaint-free residences. Applied industrial hygiene, 4(1):17-20. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

[70] Wallace L, Nelson W, Zeigenfus R, Pellizzari E, Michael L, Whitmore R, Zelon H, Hartwell T, Perritt R, Westerdahl D (1991) The Los Angeles Team study: Personal exposures, indoor-outdoor air concentrations, and breath concentrations of 25 volatile compounds. Journal of exposure analysis and environmental epidemiology, 2:157-192. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

[71] Fellin P, Otson R (1993) Seasonal trends of volatile organic compounds (VOCs) in Canadian homes. In: Saarela K, Kalliokoski P, Seppänen O, eds. Indoor air '93. Vol. 2. Chemicals in indoor air, material emissions. Proceedings of the 6th International Conference on Indoor Air Quality, held in Helsinki, 4-8 July 1993, pp. 117-122. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

arithmetic mean of limonene levels in indoor and outdoor air was reported as 0.04 and 0.002 mg/m^3, respectively, based on the study from Los Angeles (Wallace *et al*. 1991).

IPCS[72] considered exposure to limonene via drinking water is likely to be negligible due to its low solubility, although there is also a potential for dermal exposure in the occupational environment, the principle route of occupational exposure to limonene is likely to be via the inhalation route. The dermal uptake of d-limonene in humans was reported to be low compared with that by inhalation (Falk et al., 1991[73]); however, no quantitative data are provided. IPCS report data from the National Exposure Database in Norway; occupational environment concentrations of limonene between 1985 and 1992 ranged from 0 to 886 mg/m^3 (mean 28 mg/m^3) (Fjelstad and Wolbæk, 1992[74]). Occupational concentrations ranged from 0.9 to 400 mg/m^3 (Carlsson *et al*.1991[75]) in a study from Sweden,

The estimated intake of limonene from occupational exposure was determined assuming 8 of 24 hours are spent in the workplace each day, with an air concentration of 150 mg/m^3, which is the occupational exposure limit value in Sweden (National Board of Occupational Safety and Health, 1993[76]). The intake of limonene associated with working at the occupational exposure limit was estimated as 17 mg/kg body weight per day.

IARC confirm the 8 hr time-weighted exposure limit for d-limonene in Sweden is 150 mg/m^3 and the short term exposure limit is 300 mg/m^3.

IIA 5.9.4 Clinical signs and symptoms of poisoning and details of clinical tests

Specific signs of poisoning: QRD 460 is of low acute toxicity. In animal studies symptoms are indicative of general toxicity rather than a more direct effect of the chemical and are transient. The same can be expected for humans, however, no cases of intoxication with QRD 460 have yet been observed.

Clinical tests: No specific monitoring programs have been performed in humans.

IIA 5.9.5 First aid measures

General	Remove from source of exposure. If irritation or other signs of toxicity occur, seek medical attention.
In case of skin contact:	Remove contaminated clothing. Thoroughly wash skin with plenty of soap and water. If irritation persists, seek medical attention.
In case of eye contact:	Hold eye open and rinse slowly and gently with plenty of water for at least 15 minutes. If present, remove contact lenses and continue rinsing eye. Seek medical attention.
In case of ingestion:	Call a doctor immediately if a large amount is swallowed. Have person sip a glass of water if able to swallow. Do not give anything by mouth to an unconscious person.
In case of inhalation:	Move person to fresh air. If person is not breathing, call an ambulance, then give

[72] IPCS (1994). Assessing health risks of chemicals: Derivation of guidance values for health-based exposure limits. Geneva, World Health Organization, International Programme on Chemical Safety (Environmental Health Criteria 170). (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

[73] Falk A, Fischer T, Hagberg M (1991) Purpuric rash caused by dermal exposure to d-limonene. Contact dermatitis, 25:198-199. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

[74] Fjelstad PE, Wolbaek T (1992) A national exposure database. In: Brown RH, Curtis M, Saunders KJ, Vandendriessche S, eds. Clean air at work. New trends in assessment and measurement for the 1990s. Cambridge, Royal Society of Chemistry, pp. 303-310. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

[75] Carlsson H, Andersson-Sköld Y, Antonsson A-B, Solyom P (1991). [Limonene - a solution of the environmental problems.] Swedish Environmental Research Institute (Report B 1030) (in Swedish, with English summary). (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

[76] National Board of Occupational Safety and Health (1993). Occupational exposure limit value. Solna (Ordinance AFS, Vol. 3). (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

	artificial respiration.

IIA 5.9.6 Therapeutic regimes

This product has low oral, dermal, and inhalation toxicity. Direct contact with eyes may cause temporary irritation. Repeated or prolonged direct contact with skin may cause irritation in some individuals. No antidote is known, therefore, provide symptomatic and supportive care as necessary.

IIA 5.9.7 Expected effects and duration of poisoning as a function of the type, level and duration of exposure or ingestion

No specific effects can be deduced from animal data. No human data are available.

IIA 5.9.8 Expected effects and duration of poisoning as a function of varying time periods between exposure or ingestion and commencement of treatment

No specific effects can be deduced from animal data. No human data are available.

IIA 5.9.9 Dermal penetration

No studies with QRD 460 have been undertaken.

Literature sources: In shaved mice, the dermal absorption of [^{14}] d/l-limonene from bathing water was rapid and reached the maximum level in 10 minutes (von Schäfer & Schäfer, 1982[77]). In one human study where one hand was exposed to 98% d-limonene for 2 hours, the dermal uptake of d-limonene in humans was reported to be low compared with that by inhalation (Falk, et al., 1991); however, no quantitative data are provided.

The default value of 100% absorption will therefore be used for risk assessment.

IIA 5.10 Other/special studies

This is not an EC data requirement.

IIA 5.11 Summary of mammalian toxicity and overall evaluation

AgraQuest Inc. believes that, together with the studies conducted, there are sufficient data on the toxicity of the terpene components of QRD 460 to assess mammalian toxicity, and hence further studies are not required. Furthermore, use of products containing QRD 460 will not result in repeated human exposure to QRD 460 by the oral, inhalation or dermal routes.

Studies on the absorption, distribution, excretion and metabolism in animals

There are no ADME data for QRD 460, however published data exist for its terpene components d-limonene and p-cymene and these data indicate the terpenes have similar pathways of metabolism in animals and humans. Following an oral dose, the components of QRD 460 are rapidly and well absorbed from the gastrointestinal tract and metabolites are excreted, mostly via urine, within 48 hours. The amount of d-limonene absorbed via the oral route is similar in different species; reported values range from 50-96% in rats, guinea-pigs, hamsters and dogs whilst those in human male volunteers are reported as 50-80%. Absorption via the inhalation route is also rapid; the percentage absorbed is reported to average 65%.

[77] von Schäfer R, Schäfer W (1982) Die perkutane Resorption verschiedener Terpene-Menthol, Campher, Limonen, Isobornylacetat, alpha-pinen-aus badezusätzen. *Drug research,* 32:56-58. (Cited in IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene)

Whilst fewer data were identified for p-cymene, similar absorption values (60-80%) are reported in rats and guinea pigs. Given the similar structure and properties of terpinene, absorption values are likely to be comparable. The available data indicate the components of QRD 460 are readily metabolised to materials which are rapidly excreted within 48 hours.

Acute toxicity

QRD 460 is harmful if swallowed; it is of low acute toxicity by the dermal and inhalation routes. The acute oral LD_{50} in rats is 2,000 mg/kg, the acute dermal LD_{50} is >5,050 mg/kg and the acute inhalation LC_{50} in rats is > 5.30 mg/L. No gross abnormalities were noted at necropsy. QRD 460 is not irritating to the eyes or skin. QRD 460 is a skin sensitiser.

While QRD 460 did give a positive result in the LLNA test, it is important to consider that two other tests conducted with substantially similar active substances using the Buehler and Magnusson and Kligman maximization methods were negative for sensitization. It seems unusual that a simple, previously tested, mixture of the three terpene constituents would result in a positive response when the LLNA method is employed. d-Limonene has been classified as a weak sensitizer, however, this has been widely attributed to the presence of limonene oxidation products,[1] but neither α-terpene or ρ-cymene are classified as sensitizers. Literature indicates that QRD 460 does not contain sufficient d-limonene to trigger a strong sensitizing reaction, and internal investigations indicate that there are not significant amounts of limonene oxides in the test material, so the reason for the positive LLNA result is not easily explained.

The product, QRD 452, also gave a negative result for sensitization using the Buehler method. However, because the LLNA result for QRD 460 would override the Buehler result for QRD 452 in the hazard assessment, and a Calculation Rule would be applied, a LLNA test using QRD 452 was commissioned. The preliminary results indicate this test is positive; AgraQuest Inc. is investigating these new results.

In common with all toxicity tests, the LLNA is not 100% accurate, reports in the literature indicate it is approximately 90% reliable (similar to Magnusson and Kligman and Buehler), therefore, the possibility exists that the positive results reported for QRD 460 may not be indicative of the active substance's true biological nature. Potential false positives in the LLNA are not unprecedented. Other examples of materials implicated in this manner include: sodium lauryl sulfate, fatty acids such as oleic acid and linoleic acid, squalene, octinol, long-chain fatty acids, and non-ionic sugar lipid surfactants.[2]

Finally, real world experience with the plant extract-based and terpenoid blend active substances, as well as their respective formulated products, do not support the conclusions of the LLNA tests. The plant extract-based and terpenoid blend active substances have been manufactured for a number of years without a single report of dermal sensitization from manufacturing personnel. Similarly, the plant extract-based and QRD 452 plant protection products have been widely used (development trials and commercial use) in the USA with no reports of dermal sensitization or other adverse effects.

[1] Christensson JB, Johansson S, Hagvall L, Jonsson C, Börje A, Karlberg AT. (2008) Limonene hydroperoxide analogues differ in allergenic activity. Contact Dermatitis, 59: 344-352
[2] [redacted] – personal communication to [redacted] // (2011)

Short term toxicity

Whilst no data are available on QRD 460 to address short or long term toxicity, AgraQuest Inc. believe use of products containing QRD 460 will not result in repeated human exposure to QRD 460 by the oral, inhalation or dermal routes; furthermore the acute data on the active ingredient, formulation and short/long term toxicity data on the components of QRD460 indicate low toxicity. The inherent exposure of humans to the active ingredient components through their natural occurrence or via their use as permitted direct food additives, flavouring agents, and fragrances in food, cosmetics, and other consumer goods, including other pesticides, has not resulted in widespread reports in the published literature of adverse effects. α-Terpinene, p-cymene, and especially d-limonene are the most common terpenes in nature; they occur naturally in a multitude of fruits (especially citrus), vegetables, herbs, spices (cumin, neroli, bergamot and caraway), and other foods and beverages, including coffee, tea, alcoholic beverages,

baked and fried potatoes, bread and cheese. Although the levels are relatively low, the general public is exposed to these components through ingestion, dermal contact, and inhalation on a daily basis. According to a 2005 World Health Organization (WHO) report[78] on food additives, the per capita daily consumption of the three main components as food additives in the US and Europe, respectively, are: d-limonene, 12.76 mg and 39.307 mg; p-cymene, 0.472 mg and 1.085 mg; α-terpinene, 0.093 mg and 0.032 mg.

The Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC) reported Maximised Survey-derived Daily Intakes for the three terpene components. All were considered of no safety concern at the estimated levels of intake.

Genotoxicity

Three genotoxicity assays have been conducted with QRD 406 which contains essentially the same proportions of the three active terpene components as QRD 460. The reverse mutation assay showed that the tgai was not mutagenic to bacterial strains TA98, TA100, TA1535, TA1537, and *E. coli* strain WP2 *uvrAW*. The test material produced no statistically significant increases in chromosome/chromatid aberrations in human lymphocytes with, or without, metabolic activation. The third mutagenic assay was also negative; the tgai did not cause unscheduled DNA repair in cultured rat hepatocytes. One of QRD 460's components, d-limonene has also been studied in a battery of short-term *in vitro* tests; there is no evidence that the active ingredient, component or its metabolites are genotoxic. These data together with an evaluation of the three components of QRD 460 by SAR is sufficient to allow a conclusion of no significant genotoxic activity.

Long term toxicity and carcinogenicity

Whilst there are no long term or carcinogenicity data, there is no reason to anticipate any carcinogenicity due to a genotoxic action. Furthermore, the data for d-limonene indicates that after repeated administration to rats and mice, the only tumours noted were in the male rat kidney and these have been generally accepted as due to a non-genotoxic mechanism, namely through the accumulation of alpha 2U-globulin. Further use of animals in conducting long term studies on these chemicals is considered unnecessary.

Reproductive and developmental toxicity

A published report on the embryotoxicity of α- terpinene (where the developmental NOAEL was reported as 30 mg/kg based on developmental effects at 60 mg/kg bw/day whilst the maternal NOAEL was 60 mg/kg) prompted a developmental toxicity study on QRD 420. QRD 420 is structurally comparable to QRD 460 (except QRD 420 contains canola oil, see document J). The NOAEL for maternal toxicity was 60 mg/kg/day QRD 420. Developmental toxicity was seen only in the presence of maternal toxicity and a reduction in foetal weight. The NOAEL for developmental toxicity was 120 mg/kg/day for QRD 420. There was no evidence of teratogenicity or abnormal foetal development in the rat, due to maternal treatment with QRD 420 during the period of organogenesis, at dose levels that induced maternal toxicity. Minor, transient alterations in the ossification of a few areas of the foetal skeleton were seen only in the presence of maternal toxicity and reduced foetal weight.

Neurotoxicity

There is no indication, either from the data on QRD 460 or from literature sources, that the material has neurotoxic potential.

Dermal adsorption

No data have been generated.

Of the components in QRD 460, most published data are available for d-limonene. The International Programme on Chemical Safety[79] reviewed d-limonene in 1998 and considered it to "essentially non-toxic". Following oral or intraperitoneal administration, the target organ in animals (except male rats) was the liver. Exposure affects the

[78] WHO (2005), Evaluation of Certain Food Additives. WHO Technical Report Series No. 928. 63rd Report of the Joint FAO/WHO Expert Committee on Food Additives.
[79] IPCS (1998). Concise International Chemical Assessment Document No 5. Limonene

activity of different liver enzymes, liver weight, cholesterol levels and bile flow; changes have been reported in mice, rats, and dogs. d-Limonene causes a male rat-specific nephrotoxicity resulting from the accumulation of the male rat-specific alpha2μ-globulin, female rats and males of strains that do not express this protein and other species are not susceptible to the nephrotoxic action of d-limonene. Therefore the nephrotoxicity is considered not to be relevant for human risk assessment. Canada's Pest Management Regulatory Agency (PMRA)[80] has also proposed full registration for the use of d-limonene to control insects. They considered it to be of low toxicity with no evidence that it is carcinogenic to humans, neurotoxic, genotoxic, teratogenic or a reproductive toxicant.

The Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in contact with Food (AFC) considered p-cymene and α-terpinene and d-limonene along with other aliphatic, alicyclic and aromatic saturated and unsaturated tertiary alcohols, aromatic tertiary alcohols and aromatic hydrocarbons 2006[81], and 2008[82]. They were considered to be of no safety concern at the estimated levels of intake.

In summary, QRD 460 shows low acute toxicity and whilst no data exist for repeated exposures, given the use patterns for this product, no exposure is expected. Data on one of the components of QRD 460 also indicates low toxicity following repeat exposure.

Overall:

- Repeated human exposure by the oral route is not anticipated for QRD 460 or its end use product QRD 452.
- The opportunity for exposure is small, given the limited number of applications and the interval between applications. The components of QRD460 are rapidly excreted, therefore any exposure would be a series of acute events rather than subchronic in nature.
- A residue decline study showed that the marker components of the active ingredient were not detectable 10 minutes after application to primrose leaves.
- A study where QRD 400 was applied four times at ~2 kg as/ha on tomatoes found that the residues of marker components were below the limit of what can be measured (0.01 mg/kg) at 0 to 24 hrs post-treatment.
- The components of QRD 460 have all been approved by FDA and WHO as food additives.

Acceptable daily intake (ADI)

The acceptable daily intake (ADI) is derived from the NO(A)EL in the most susceptible species in long term toxicity and multi-generation reproduction toxicity studies with the application of an appropriate safety factor. The dietary route of exposure is the most relevant for derivation of this end-point

However, it has been established that there will be no detectable residues of the marker components of the active ingredient on any raw agricultural commodities at the time of harvest resulting from the use of products containing QRD 460.

The components of QRD 460 (α-terpinene, p-cymene, and d-limonene) are naturally occurring in a multitude of fruits, vegetables, herbs, spices, and other foods and beverages. Although the levels are relatively low, the general public is exposed to these components through ingestion, dermal contact, and inhalation on a daily basis. According to a 2005 World Health Organization (WHO) report[83] on food additives, the per capita daily consumption of the three main components as food additives in the US and Europe, respectively, are as follows: d-limonene, 12.76 mg and 39.307 mg; p-cymene, 0.472 mg and 1.085 mg; α-terpinene, 0.093 mg and 0.032 mg.

[80] Health Canada. Proposed Registration Decision PRD2010-21, d-Limonene. 27 Aug 2010. http://www.hc-sc.gc.ca/cps-spc/pest/part/consultations/_prd2010-21/d-limonene-eng.php

[81] Opinion of the Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC) related to Flavouring Group Evaluation 18 (FGE.18): Aliphatic, alicyclic and aromatic saturated and unsaturated tertiary alcohols, aromatic tertiary alcohols and their esters from chemical group 6 (2006). The EFSA Journal 331, 1-77.

[82] Flavouring Group Evaluation 25, (FGE.25)[1] - Aliphatic and aromatic hydrocarbons from chemical group 31 - Scientific Opinion of the Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (2008). The EFSA Journal 918, 1-109.

[83] WHO (2005), Evaluation of Certain Food Additives. WHO Technical Report Series No. 928. 63rd Report of the Joint FAO/WHO Expert Committee on Food Additives.

The Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC) reported MSDI (Maximised Survey-derived Daily Intakes) values for p-cymene of 926 ug/capita/day[84], α-terpinene of 27 ug/capita/day[85] and d-limonene of 33542 ug/capita/day[93]. All were considered of no safety concern at the estimated levels of intake. In the EU, JECFA considered d-limonene poses no safety concerns at the estimated current intakes in Europe. The establishment of an acceptable daily intake expressed in numerical form was not deemed necessary.

In conclusion use of plant protection products containing QRD 460 will not contribute to dietary exposure of these terpene components and it is therefore not relevant to establish an ADI for QRD 460.

This is consistent with the regulatory situation in the US where the EPA granted exemption from the requirement for a tolerance (40 CFR 180.1296) based on absence of detectable residue and resultant lack of oral exposure to all populations.

Acute Reference Dose (ARfD)

On the basis of the available database on QRD 460 and its terpene components no acute reference dose is required.

- QRD 460 shows low acute oral toxicity with an MLD of 2000 mg/kg.
- A rat oral developmental toxicity study on QRD 420 revealed no teratogenic or embryotoxic effects in the absence of maternal toxicity.
- There were no clinical signs indicative of pharmacological or target organ toxicity in the studies conducted with QRD 460.
- There is no indication, either from the data on QRD 460 or from literature sources, that the material has neurotoxic potential.
- All available information supports that QRD460 is not comprised of estrogenic, anti-estrogenic, androgenic, or anti-androgenic components.

Acceptable Operator Exposure Level (AOEL)

The acceptable operator exposure level (AOEL) is derived from the NO(A)EL in the most susceptible species in short-term toxicity (including dermal studies as this route is particularly relevant) and multi-generation reproduction/developmental toxicity studies with the application of an appropriate safety factor.

Whilst no data are available on QRD 460 to address short or long term toxicity, AgraQuest Inc. (AQ) believe use of products containing QRD 460 will not result in repeated human exposure to QRD 460 by the oral, inhalation or dermal routes (see section IIA 5.3 for full details). Furthermore the acute data on the active ingredient, formulation and short/long term toxicity data on the components of QRD 460 indicate low toxicity.

A rat developmental toxicity study was conducted with QRD 420 to better understand possible embryofetoxic effects from α-terpinene reported in the open literature. The NOAEL for maternal toxicity was 60 mg/kg/day QRD 420. Developmental toxicity was seen only in the presence of maternal toxicity and a reduction in foetal weight. The NOAEL for developmental toxicity was 120 mg/kg/day for QRD 420. There was no evidence of teratogenicity or abnormal foetal development in the rat, due to maternal treatment with QRD 420 during the period of organogenesis, at dose levels that induced maternal toxicity. Minor, transient alterations in the ossification of a few areas of the foetal skeleton were seen only in the presence of maternal toxicity and reduced foetal weight. However, in the absence of context from other studies relevant for AOEL setting it is not considered appropriate to use this single study as a basis for setting an AOEL.

It can therefore be concluded that it is not relevant to establish an AOEL for QRD 460.

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84 Opinion of the Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC) related to Flavouring Group Evaluation 18 (FGE.18): Aliphatic, alicyclic and aromatic saturated and unsaturated tertiary alcohols, aromatic tertiary alcohols and their esters from chemical group 6 (2006). The EFSA Journal 331, 1-77.

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IIA 5.2.1/01	[redacted] J	2011a	QRD 460: Acute oral toxicity study (UDP) in rats. [redacted]. Laboratory Report No. 14695-10, 01 March 2011. GLP, Not published.	Y	AQ
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