ELSEVIER

Food Chemistry 84 (2004) 643–649

Food Chemistry

www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods Section

The analysis of honey samples for residues of nitrobenzene and petroleum from the possible use of Frow mixture in hives

Laurence Castle*, Mark R. Philo, Matthew Sharman

Central Science Laboratory, Department for Environment, Food and Rural Affairs, Sand Hutton, York YO41 1LZ, UK

Received 2 February 2003; received in revised form 16 June 2003; accepted 1 July 2003

Abstract

A headspace gas chromatography–mass spectrometry method has been established for the analysis of honey samples for nitrobenzene and petroleum residues. These substances may be used as components of 'Frow mixture' applied to treat hives for the control of Acarine (infestation by parasitic mites) in honey bees. The method had a detection limit of 2 μg/kg for nitrobenzene and 0.5 μg/kg (or better) for four indicator substances of petroleum—toluene, *o*-xylene, ethylbenzene and naphthalene. The method was applied in a small survey of 49 retail honey samples. No sample contained detectable nitrobenzene or petroleum residues defined as the presence of all four indicator hydrocarbons. Two honey samples contained low levels of toluene (19 and 62 μg/kg) and one of these two samples also contained *o*-xylene (2 μg/kg). The origin of these very low levels was not investigated. These analytical findings indicate that, for honey samples entering the UK market, either the use of Frow mixture to fumigate bee hives is not commonly practiced or that chemical residues from the treatment are not persistent.

Keywords: Honey; Frow mixture; Nitrobenzene; Petroleum; Contamination; Headspace GC–MS

1. Introduction

This work was conducted as part of the UK non-statutory programme to monitor food samples for residues of veterinary medicines. According to statistics from the Food and Agriculture Organisation of the United Nations (FAO, 2003), world honey production in 2001 was 1,264,000 tonnes. This is a minimum figure since honey production data are incomplete, particularly with regard to African and Asian countries. About 25% of this production enters international trade.

There is a possibility that residues of nitrobenzene and petrol may be found in honey because of the occasional use of these substances to control Acarine in honeybees. Acarine is the term commonly used to denote an infestation of the adult honeybee by a parasitic mite inhabiting the breathing tubes of the thorax (Ministry of Agriculture Fisheries & Food, 1982). Acarine can threaten the viability of a hive. One method to treat Acarine is to fumigate with volatile substances or aerosols while the bees are confined to the hive. A traditional mixture used for this purpose is the volatile Frow mixture. Other 'traditional' treatments of tracheal mites involve volatile essential oils, including wintergreen or menthol. Commercial treatments include, in some countries, smoking with a smouldering strip containing bromopropylate, which controls varroa mites as well as tracheal mites. Fumigation by amitraz is another treatment that has been reported (Dag et al., 1997).

2. Frow mixture composition and usage

Frow mixture comprises nitrobenzene (two parts v/v), safrole (one part) and petrol (also known as gasoline) or ligroin (two parts). Typically, 2 ml of the Frow mixture is poured onto a flannel pad which is placed over the feed hole of the hive so that the vapour mixture permeates the hive. This is repeated every other day to give seven doses in all. If repeated visits cannot be made then a single dose of ca. 4.5 ml can be applied but there is some possibility of killing the colony as a result of too high a concentration of the vapour—especially if the weather is mild.

* Corresponding author. Tel.: +44-1904-462540; fax: +44-1904-462133.

E-mail address: l.castle@csl.gov.uk (L. Castle).

0308-8146/$ - see front matter
doi:10.1016/j.foodchem.2003.07.027

Information from the UK National Bee Unit at CSL is that Frow mixture is not commonly used nowadays to control Acarine. If it is used, it is normally applied only in the late autumn (in the UK, October/November depending on latitude) or early spring (February/March) since it is likely to induce robbing at times when the bees are active and may disturb the cluster if used during winter. Because some weeks or months will elapse between any use of Frow and the productive honey-making period, it is likely that much if not all of any Frow volatiles would have by then dissipated from the hive structure. Nevertheless, there may be residues that remain. There is also the possibility that in countries other than the UK the use of Frow is more widespread and at different times relative to the foraging season.

3. Target substances and their level of interest

Safrole is a natural toxicant, one of a family known as biologically active principles (BAPs). It belongs to the class of alkyl-alkoxybenzene derivatives comprising, among others, the BAPs estragole, safrole, methyleugenol, eugenol and myristin. As the exemplar of the group, estragole has been demonstrated to be genotoxic and carcinogenic (Scientific Committee on Food, 2001). Safrole can be found in a variety of food products as a result of its presence in natural flavouring source materials. For example, cinnamon and nutmeg oils contain safrole. Limits on the levels of safrole permitted in food as a result of their presence in these flavourings are set in EC Directive 88/388/EEC. The regulatory limit for safrole in food is 1 mg/kg, with specified exceptions for some alcoholic beverages and foods containing mace and nutmeg. Safrole was not analysed for in the honey samples.

For the two man-made chemical constituents of Frow mixture, neither nitrobenzene nor petrol are specifically permitted pesticides for honey production. The maximum residue limit (MRL) is 'not set' (Veterinary Medicines Directorate, 2002). The Residues Regulations would prohibit the administration of these non-medicinal curative substances only if they are transmitted to honey at levels likely to be harmful to human health. As a precautionary measure, the level of interest was set for this work as not detectable using a method with a limit of detection (LoD) of 10 μg/kg.

4. Analytical approach

The most straightforward analytical approach for moderately volatile substances in foodstuffs is direct (also known as static) headspace gas chromatography coupled to mass spectrometry (HS-GC–MS). As it was anticipated to have sufficient sensitivity to attain the required LoD of 10 μg/kg then this was the approach used here.

There are over 500 hydrocarbon components that can be found in petrol and the exact composition will depend on the type of petrol (e.g. unleaded, premium, 4-star and city) and the combination of hydrocarbons that the manufacturer blends to give the octane rating required. The composition will also be varied according to the location and season to suit the prevailing conditions of altitude and temperature. Lastly, the composition will be changed according to the spot market price of the ingredients.

As an example of this complexity, a 'typical' petrol might contain

15% *n*-paraffins (C_4–C_8)
30% *iso*-paraffins (C_4–C_8)
12% cycloparaffins (C_5–C_7)
35% aromatics (C_6–C_9)
8% olefins (C_5–C_6)

The use of oxygenated fuels (e.g. methyl-*t*-butyl ether) at up to about 12–15% reduces the aromatics and olefins content.

This complex composition posed the question—exactly what should be looked for in honey as evidence of any contamination by petrol? The lower hydrocarbons are more fugitive (volatile) and so might be expected to contaminate more. But they should also dissipate more quickly from the hive, perhaps leaving behind an over-representation of the higher hydrocarbon fraction. Thus, for example, the aromatics xylenes, alkylbenzenes, naphthalene and alkyl naphthalenes do not represent the most abundant classes of gasoline components but they are the most persistent after evaporation of the mixture (Rella, Sturaro, Parvoli, Ferrara, & Doretti, 2002). It was decided therefore to monitor the hydrocarbons in Table 1 as key petrol components along with the nitrobenzene component of Frow mixture.

5. Materials

5.1. Chemicals

Primary standards of the analytes naphthalene (99 + %), nitrobenzene (99 + %), ethylbenzene (99 + %) and *o*-xylene (98%), the internal standards d_5-nitrobenzene (99.5%), d_8-toluene (99.96%), d_{10}-ethylbenzene (98%) and d_{10}-*o*-xylene (99 + %), and the solvent dimethylacetamide (99.9 + %), were purchased from Aldrich Chemicals Co. Ltd. (Dorset, UK). The analyte toluene (HPLC grade) was from Fisher Scientific UK Ltd. (Leicester, UK). Samples of unleaded and lead replacement

Table 1
The selected indicator components of Frow mixture and their boiling points

Petrol component (40% v/v in Frow)				Nitrobenzene component (40% v/v in Frow)
Toluene	Ethylbenzene	*o*-Xylene	Naphthalene	Nitrobenzene
111 °C	136 °C	144 °C	218 °C	211 °C

petrol were purchased from a local filling station. Portions were placed in dishes in a fumecupboard and allowed to evaporate over several days at room temperature to achieve an 80% reduction in volume.

5.2. Honey samples

For method development purposes, four jars of honey were purchased from local stores. Two were clear honeys (Australian Eucalyptus and a honey blend of mixed origin) and two were creamed honeys (from Wales and from Scotland). For survey purposes, 49 samples of honey were received from UK border inspection posts and retail shops. They comprised clear, creamed and combed varieties; 26 were products of the UK and 23 were imported honeys from New Zealand (five samples), China (4), Australia (4), Argentina (3), Cuba (2), Greece (1), Mexico (1) and 'undeclared' (3). These honey samples had also been tested for other contaminants, including lead (detected in nine of the 49 samples, range 25–52 μg/kg) and streptomycin (detected in four of the 49 samples, range 100–210 μg/kg) (Veterinary Medicines Directorate, 2002).

6. Methods

6.1. Sampling

Samples were prepared in a clean room away from laboratory solvents. A portion of honey (5 g) was placed in a head space vial (10 ml capacity). d_5-Nitrobenzene, d_8-toluene, d_{10}-ethylbenzene and d_{10}-*o*-xylene were added as internal standards (final concentration 80 μg/kg) dissolved in a small volume of dimethylacetamide (10 μl). For calibration purposes, two types of honey, one clear and one creamed, were spiked with nitrobenzene and with weathered unleaded petrol dissolved in dimethylacetamide, to cover the concentration range of 0–120 μg/kg. The headspace vials were capped and placed on a horizontal roller mixer for 30 min to ensure complete incorporation of the internal standards and the calibration standards.

6.2. Analysis

The samples were incubated at 90 °C for 45 min and a portion of the headspace gas (1 ml) then analysed by GC–MS. The head space portion was injected in split mode (250 °C, 15:1 split ratio) onto a capillary column coated with a 14% cyanopropylphenyl/86% methyl polysiloxane stationary phase (50 m × 0.32 mm i.d., 1.2 μm phase thickness). Helium was used as carrier gas at 1 ml/min. Following injection, the column was held at 40 °C for 1 min, then raised at 8 °C/min to 300 °C and held for 10 min. Mass spectrometric detection was conducted in selected ion electron impact mode. The ions monitored are listed in Table 2.

7. Results and discussion

7.1. Method performance

7.1.1. Criteria for confirmation of detection

MS scanning analysis of the petrols revealed an array of aliphatic and aromatic substances. A comparison of fresh to weathered petrols indicated the expected loss of the more volatile components with the aromatics remaining prominent. Fig. 1 shows a GC–MS chromatogram for a weathered unleaded petrol. A replacement-leaded petrol showed a similar profile except with lesser amounts of naphthalene and fluorene derivatives.

The individual hydrocarbons that were used as indicators of the presence of petrol residues were toluene, ethylbenzene, *o*-xylene and naphthalene (Table 1). For any one of these individual hydrocarbons to be confirmed as detected, and for nitrobenzene to be confirmed as detected, the following criteria were applied:

Table 2
Selected ions monitored in HS-GC–MS

Substance	Ion(s) monitored
Analytes	
Toluene	77, 91
Ethylbenzene	77, 91, 106
o-Xylene	77, 91, 106
Naphthalene	128
Nitrobenzene	77, 123
Internal standards	
d_8-toluene	99
d_{10}-*o*-Xylene	116
d_{10}-Ethylbenzene	116
d_5-Nitrobenzene	128

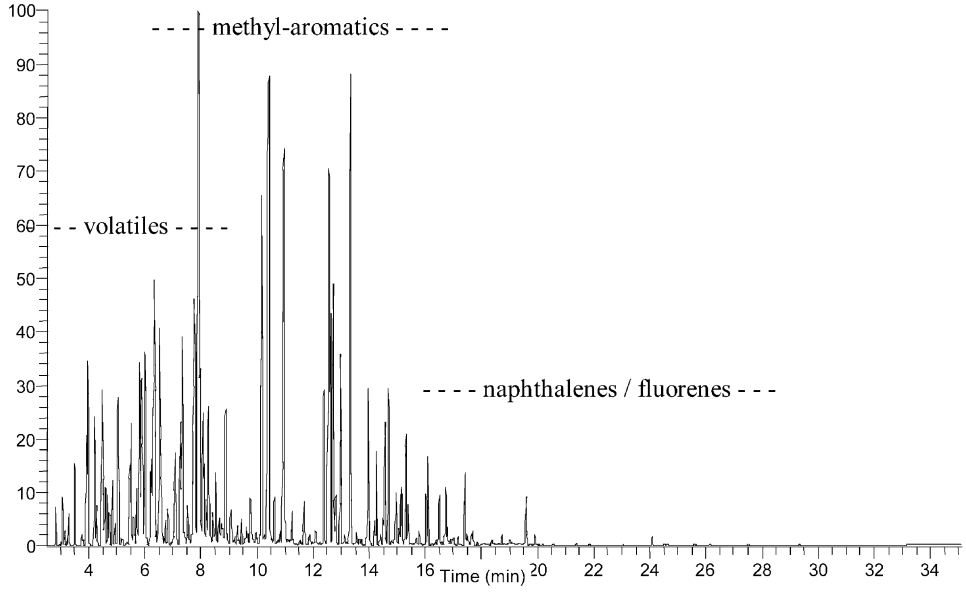

Fig. 1. HS-GC–MS total ion chromatogram of weathered unleaded petrol.

- the signal for the substance in a sample must be no less than 3 times the noise of the average of the blanks run in that analytical batch;
- the ions listed in Table 2 for that substance must maximise within 3 s of the retention time for that substance detected in the calibration standards and in the spiked samples; and
- the ratio of the ions listed in Table 2 for that substance must agree to within 20% of the ratio for that substance detected in the calibration standards and in the spiked samples.

Because no suitable secondary ion was present in the mass spectrum of naphthalene, the third criteria was excluded for this substance.

For petrol residues to be confirmed as being detected, an additional criteria had to be satisfied after confirmation of the presence of its marker hydrocarbons.

- toluene, *o*-xylene, ethylbenzene and naphthalene should all be detectable in the sample, although the exact proportions were not fixed (because of possible weathering).

7.1.2. Limit of detection

The limit of detection of the HS-GC–MS method, defined as three times the noise of the analytical batch blanks, was 0.5 μg/kg or better for each of the four individual petrol hydrocarbons and was less than 2 μg/kg for nitrobenzene. A chromatogram of a honey sample spiked with 2.5 μg/kg of each substance is shown as Fig. 2.

For samples of honey spiked with the individual substances, the MS response was linear over the range 2.5–120 μg/kg with correlation coefficients of at least 0.997. For samples of honey spiked with weathered petrol, the MS response to the four targeted hydrocarbon components was linear over the range 10 to 400 μg/kg with correlation coefficients of at least 0.950.

The limit of detection for petrol, expressed as a mixture and detected by the four component hydrocarbons, was 10 μg/kg. Figs. 3 and 4 show the chromatogram from a honey sample spiked with petrol at 100 μg/kg. The more volatile aromatics are prominent (Fig. 3) and even the substituted naphthalenes and fluorenes—which are less abundant and less volatile in the headspace method—can be seen clearly (Fig. 4).

7.1.3. Choice of honey types for establishing method performance

There are three common physical states of natural honey and it was considered possible that this might have a small influence on the performance of the analytical method. The three states are liquid, granulated and thixotropic. 'Creamed' honey is made by mixing semi-granulated and re-liquefied honeys together to form a creamy consistency that will not go solid again. The thixotropic honey type only has one representative in the UK and that is heather honey, a premium honey produced from *Calluna vulgaris*. Two calibration series were prepared in a clear and in a creamed honey. The slopes of the two calibration lines were identical. It can be concluded that, because deuterated internal standards were used for the HS-GC–MS method, these give as near perfect correction for analytical recovery as is possible. Consequently the effect of the exact composition and physical form of honey on the analysis was unimportant. The analytical results were automatically

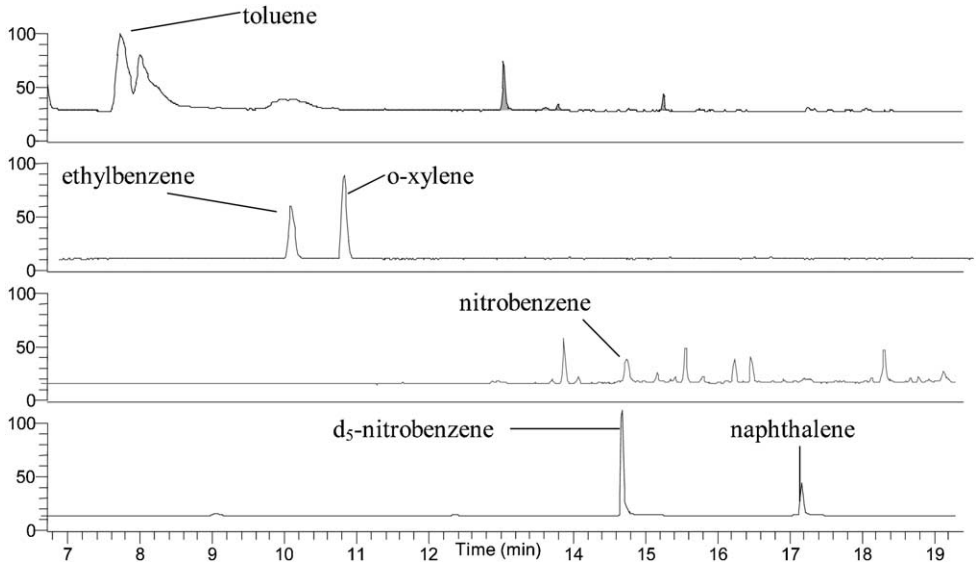

Fig. 2. HS-GC–MS selected ion monitoring traces of individual standards in honey (2.5 μg/kg).

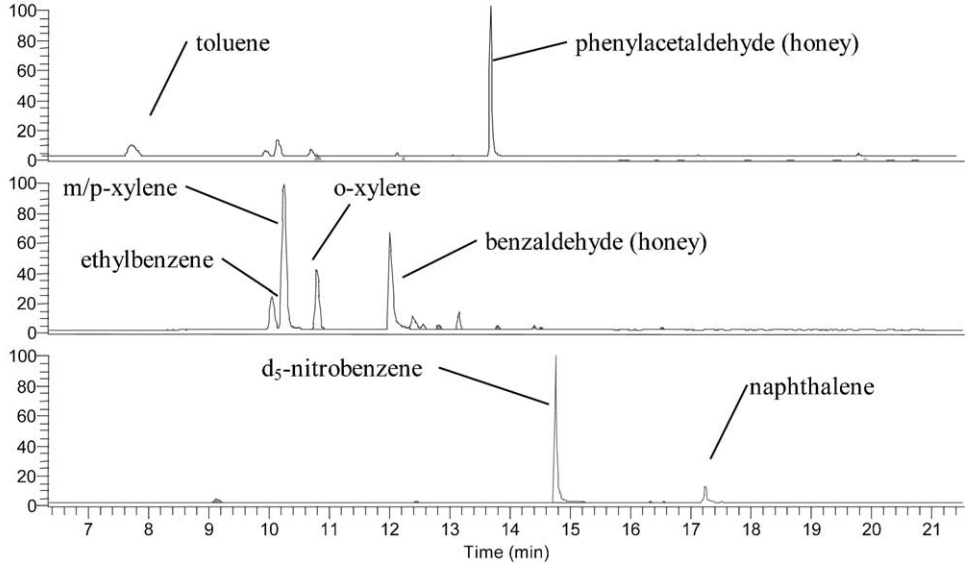

Fig. 3. HS-GC–MS selected ion monitoring traces of petrol standard in honey (100 μg/kg): volatile aromatics.

corrected for recovery, because matrix-matched calibration standards were used.

7.2. Findings

7.2.1. Analysis for nitrobenzene

None of the 49 honey samples surveyed contained a detectable residue of nitrobenzene, at a limit of detection of 2 μg/kg.

7.2.2. Findings from the analysis for petrol residues

Two of the 49 samples surveyed contained toluene. Sample 19 contained 62 μg/kg toluene but did not contain any detectable (<0.5 μg/kg) ethylbenzene, *o*-xylene or naphthalene. Consequently, the toluene that was detected did not originate from petrol and must have had another source. Toluene is used widely as a solvent in printing inks and adhesives for example. The pick-up of toluene and other hydrocarbons has been reported for confectionery sold in newsagents alongside newspapers and magazines, and in foodstuffs sold in shops on or nearby petrol filling stations (Ministry of Agriculture Fisheries and Food, 1996). Similarly, 234 table-ready foods of the FDA Total Diet Program were analysed for volatile organic compounds (Heikes, Jensen, & Flemingjones, 1995). A total of 77 foods showed residues >50 μg/kg and 43 foods had residues >100 μg/kg. Toluene was the most common residue encountered, with detectable residues in 91 (39%) of the foods that these workers tested.

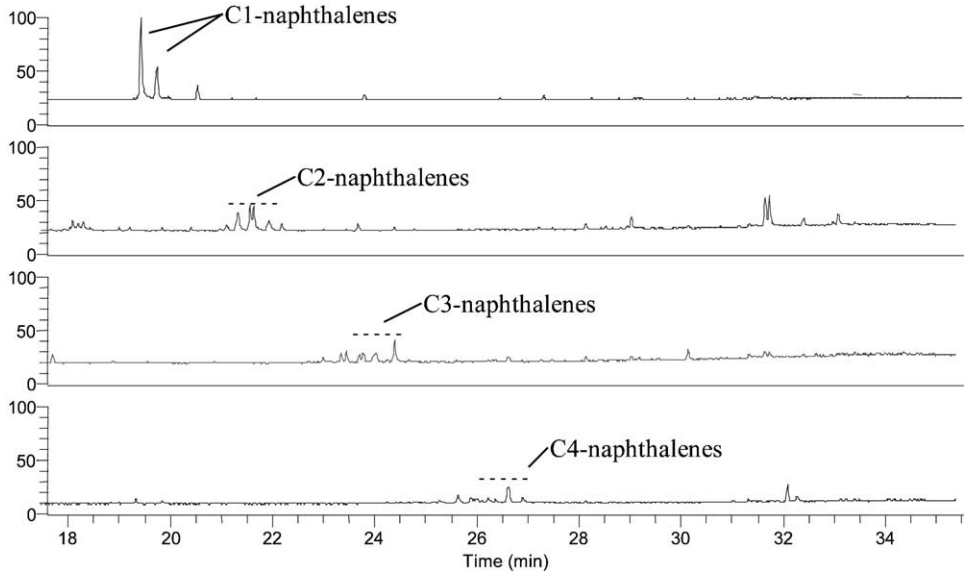

Fig. 4. HS-GC–MS selected ion monitoring traces of petrol standard in honey (100 μg/kg): naphthalenes and fluorenes.

Sample 21 also contained detectable toluene, at 19 μg/kg. This sample contained detectable *o*-xylene too, which was estimated to be 2 μg/kg. However, the sample did not contain any detectable ethylbenzene or naphthalene. Consequently, the toluene and *o*-xylene that were detected could not have originated from petrol and they must have had another source. Potential sources of toluene were discussed above. The level of *o*-xylene was very low and no obvious sources can be suggested.

With these two exceptions, none of the honey samples contained detectable amounts of the marker components for petrol. It can be concluded that none of the 49 honey samples surveyed contained a detectable residue of petrol, at a limit of detection of 10 μg/kg.

7.3. Discussion of findings

In related work on honey analysis, the volatile (aroma) profile of honeys has been recorded to look for marker compounds for authenticity purposes such as floral and geographical origin (Radovic et al., 2001). A headspace purge-trap GC–MS method was used to establish the presence or absence of 110 volatile substances in 43 authentic honey samples. The data presented by these authors were qualitative only and no cut-off concentration was given to define the boundary between 'present' and 'absent'. The internal standard used (dodecene) was added at 200 μg/kg which gives some idea of the sensitivity of the analysis. Nitrobenzene and petrol indicator substances were not detected.

Honey is composed (Food Standards Agency, 2002) mainly of a variety of sugars (76%), other carbohydrates (6%), water (18%). proteins, amino acids, vitamins and minerals (0.5%) along with traces of pollen. Consequently, there should be little affinity for the non-polar hydrocarbon components of petrol to contaminate honey. Nitrobenzene is more polar and, for example, is slightly soluble in water at ca. 2 g/l. These analytical findings indicate that either the use of Frow mixture to fumigate bee hives is not commonly practiced or that chemical residues of the treatment are not persistent.

Acknowledgements

We gratefully acknowledge financial support from the Veterinary Medicines Directorate, Ministry of Agriculture Fisheries and Food (now the Department for Environment, Food and Rural Affairs). The statements and conclusions in this paper are the responsibility of the authors alone and they should not be taken to represent the opinion of VMD or of DEFRA.

References

Dag, A., Slabezki, Y., Efrat, H., Kamer, Y., Yakobson, B. A., MozesKoch, R., & Gerson, U. (1997). Control of honey bee tracheal mite infestations with amitraz fumigation in Israel. *American Bee Journal*, *137*, 599–602.

Food and Agriculture Organisation of the United Nations. (2003). Available: http://apps.fao.org/page/collections?subset = agriculture.

Food Standards Agency. (2002). *McCance and Widdowson's the composition of foods* (6th Summary ed.). Cambridge: Royal Society of Chemistry.

Heikes, D. L., Jensen, S. R., & Flemingjones, M. E. (1995). Purge-and-trap extraction with GC-MS determination of volatile organic-compounds in table-ready foods. *Journal of Agricultural and Food Chemistry*, *43*, 2869–2875.

Ministry of Agriculture Fisheries and Food. (1982). *Acarine. MAFF leaflet* 330 (amended 1982).

Ministry of Agriculture Fisheries and Food. (1996, October). *Hydrocarbons in foods from shops in petrol stations and stalls or shops in busy roads*. Food surveillance information sheet no. 98. London: MAFF.

Radovic, B. S., Careri, M., Mangia, A., Musci, M., Gerboles, M., & Anklam, E. (2001). Contribution of dynamic GC-MS analysis of aroma compounds to authenticity testing of honey. *Food Chemistry*, *72*, 511–520.

Rella, R., Sturaro, A., Parvoli, G., Ferrara, D., & Dorett, L. (2002, September). Accelerant identification in fire debris by TCT-GC-MS (thermal desorption cold trap-GC-MS). *LC.GC Europe*, 603–609.

Scientific Committee on Food. (2001). *Opinion on Estragole (1-allyl-4-methoxybenzene)*. Adopted on 26 September 2001. DG-SANCO, Brussels document SCF/CS/FLAV/FLAVOUR//6 ADD 2 FINAL.

Veterinary Medicines Directorate. (2002). *Non-statutory surveillance results*. Available: http://www.vmd.gov.uk/mavis/nstat.pdf.