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Simultaneous Distillation-Extraction GC-MS Method for the Determination of Biologically Active Principles in Foods and Beverages

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1. SAFETY

This method involves the use of several hazardous chemicals and procedures likely to produce an increased risk to the operator. A COSHH assessment should be written to identify the risks associated with this method.

General precautions

Protective clothing including laboratory coat (buttoned), safety spectacles and gloves should be worn at all times.

Samples should be regarded as a biological hazard. Direct contact with skin is best avoided and proper attention to hygiene must be maintained.

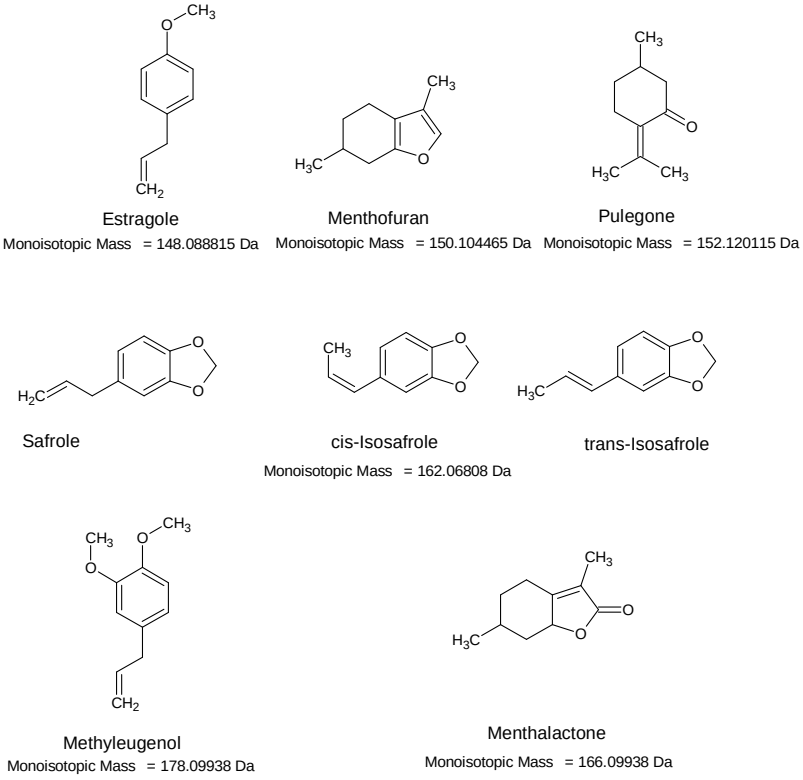
First Aid

Any injury must be reported, in the first instance, to a qualified First Aider and recorded. The First Aider will decide on further action. All accidents, incidents and near misses should be reported to the Health and Safety team.

2. INTRODUCTION

Biologically active principles (BAPS) are undesirable chemical substances that are present naturally in some flavourings and food. New EU Regulations set numerical limits on specific BAPs in food and beverages. Among these are the volatile BAPS (Figure 1).

Figure 1: Structures of volatile BAPs, isosafrole and menthalactone.



3. SCOPE

This method describes a method for the determination of volatile BAPs using simultaneous distillation extraction (SDE) and subsequent separation and detection using GC-MS in raw and processed food and beverage products with an LOQ of 0.1 mg/kg or better.

4. PRINCIPLE

The BAPs are extracted from foods and beverages using SDE into DCM. The DCM distillate is analysed by GC-MS in single-ion monitoring (SIM) mode.

5. SAMPLING

Samples are homogenised and sub-sample taken and stored at an appropriate temperature.

6. EXTRACTION APPARATUS

6.1. Glassware and Disposable Plastic Ware

General laboratory glassware to be used except where stipulated. Unless otherwise stated volumetric glassware should be of grade 'A' quality. Laboratory equipment may be generic, e.g., balances and homogenization equipment.

6.2. Equipment

Balance, 4-place (0.0001g) analytical
Balance, 2-place (0.01g) top-pan
Food mixer/blender, domestic or Waring type
Homogeniser, Ultra-Turrax with 18mm probe or equivalent
Sample shaker; AQS manufacturing Ltd., Cat. No. R100B, or equivalent
Ultrasonic bath
Water bath set at 70 ± 2 ℃
Chiller/cooler set at 5°± 2 ℃
Heating mantle for 100mL round-bottomed flasks
Positive displacement pipettes e.g. Gilson Microman M25, M50, M250 and M1000 or equivalent
Vials, glass 10 mL, screw cap
Vials, to use with GC autosampler 2 mL glass with 300 uL insert, crimp cap.

6.3. Simultaneous Distillation Extraction Apparatus

Microsteam distillation kit for solvent with density higher than water (configuration APP.2) e.g. Varian Chrompack (VWR International cat No. VARI16054) or similar. Comprising:

Microsteam distillation apparatus APP.2
Pear shaped solvent flask, 5mL, ground-glass joint size 14.5

Round-bottomed sample flask, 100mL, ground-glass joint size 14.5
Cold-finger condenser, ground-glass joint size 14.5
Teflon sleeves (3), joint size 14.5
Aluminium foil, domestic grade

6.4. Cleaning of glassware

All SDE glassware including flasks but not the condenser should be cleaned thoroughly with hot dilute detergent and rinsed with distilled water followed by acetone. To remove traces of volatile organics, the SDE glassware should be rinsed with distilled water followed by acetone, air dried then heated in an oven at *ca.* 200℃ for a minimum of 1 hour and cooled before use.

6.5. GC-MS

Capillary GC-MS system comprising:

Auto sampler with syringe wash facility
Sample volume: 1μL
Air volume: 2 μL
Sample cleans: 3
Post injection washes (DCM): 3

Split/splitless injector: Split mode
Temperature: 250℃
Split flow: 10mL/min
Split ratio: 10:1

Column: 30m x 0.25mm i.d x 0.25 μm df 5% polysilarylene 95% polydimethylsiloxane for use with MS e.g. Zebron 5-MS (Phenomenex) or similar

Carrier gas: Helium at 1 mL/min constant flow

Temperature programme
Initial temperature: 80℃
Initial time: 5 min
Ramp 1: 5℃/min to 200℃
Ramp 2: 30℃/min to 325℃ hold 5 min
Equilibration time: 0.5 min

Detection: Mass spectrometric at 70eV

SIM channels:

Mass No.	m/z	Mass No.	m/z	Mass No.	m/z
1	67	8	121	15	152
2	103	9	123	16	162
3	104	10	131	17	163
4	105	11	134	18	150
5	108	12	137	19	178
6	109	13	148		
7	119	14	150		

Qualitative identifier ions (**most abundant ion**)
Menthofuran (**108**, 150,109)
3-Methyl acetophenone (**119**, 134) Internal Standard
Estragole (**148**,121, 105)
Pulegone (**152**, 109, 137)
Propyl benzoate (**105**, 123) Internal Standard
Safrole (**162**, 131, 104)
Isosafrole (**162**, 131, 104)
Methyl eugenol (**178**, 163, 103)

Data processing software e.g. Voyager Xcalibur or similar. A sample chromatogram is shown in Figure 3.

7. REAGENTS

7.1. Chemicals

Cyclohexane, HPLC grade
Dichloromethane, HPLC grade
Water, HPLC grade
Boiling chips, fine. Rinsed with acetone, air dried and heated at ca. 200℃ for a minimum of 1 hour. After cooling, store in an air-tight container.
Propyl benzoate ≥99%, Sigma-Aldrich No. 307009 or equivalent.
3′-methyl acetophenone ≥98%, Sigma-Aldrich No. M26607 or equivalent.

7.2. BAPS:

Menthofuran ((R)-3,6-Dimethyl-4,5,6,7-tetrahydrobenzofuran) ≥99%, Sigma-Aldrich No. 63661 or equivalent.

Estragole (4-Allylanisole) ≥98.5%, Sigma-Aldrich No. 05818 or equivalent.

Pulegone ((R)-2-Isopropylidene-5-methylcyclohexanone) ≥98.5%, Sigma-Aldrich No. 82569 or equivalent.

Safrole (4-Allyl-1,2-methylenedioxybenzene) ≥97%, Sigma-Aldrich No. S9652 or equivalent.

Isosafrole (Isosafrol, *cis* + *trans*) ≥97%, Sigma-Aldrich No. 329606 or equivalent.

Methyl eugenol (4-Allyl-1,2-dimethoxybenzene) ≥99%, Sigma-Aldrich No. 284424 or equivalent.

8. STANDARDS

8.1. Stock standard solutions 5000 mg/L

Prepare single stock standard solutions of ca. 5000 mg/L of BAPs and internal standards by weighing out ca. 100 mg of standard into separate 20 mL volumetrics

and diluting to the mark with DCM *except for menthofuran*, in which case cyclohexane must be used.

8.2. Menthofuran working solution 250 mg/L

Prepare a working solution of ca. 250 mg/L by pipetting 2.5 mL of menthofuran stock standard solution (8.1) into a 50 mL volumetric and diluting to the mark with cyclohexane.

8.3. Menthofuran working dilution 25 mg/L

Prepare a working dilution of ca. 25 mg/L by pipetting 5 mL of menthofuran working solution (8.2) into a 50 mL volumetric and diluting to the mark with cyclohexane.

8.4. Mixed BAP working solution 250 mg/L

Prepare a mixed BAPs working solution of ca. 250 mg/L by pipetting 2.5 mL of the BAP working solution (8.1) into a 50 mL volumetric and diluting to the mark with DCM.

8.5. Mixed BAP working dilution 25 mg/L

Prepare a mixed BAPs working solution of ca. 25 mg/L by pipetting 5 mL of the BAP working solution (8.4) into a 50 mL volumetric and diluting to the mark with DCM.

8.6. Internal standard working solutions 250 mg/L

Prepare single working standard solutions of ca. 250 mg/L of each internal standard by pipetting 5 mL of each stock internal standard standard solution (8.1) into separate 100 mL volumetrics and diluting to the mark with DCM.

8.7. Calibration standards for GC-MS

Prepare the following dilutions in 5 mL volumetric flasks for GC-MS calibration using DCM:

Nominal conc (mg/L)	Vol 25 ug/mL mixed standard (uL)	Vol 250 ug/mL mixed standard (uL)	Vol 25 ug/mL menthofuran standard (uL)	Vol 250 ug/mL menthofuran standard (uL)	Vol 250ug/mL propylbenzoate IS standard (uL)	Vol 250ug/mL 3-MA IS standard (uL)
0	0	0	0	0	100	100
0.5	100	0	100	0	100	100
1.0	200	0	200	0	100	100
2.5	0	50	0	50	100	100
5.0	0	100	0	100	100	100
10.0	0	200	0	200	100	100

Plot the peak area ratios (BAP/PB) derived from the calibration standards against BAP concentration. Determine the regression equation for the graph from which the correlation coefficient should be 0.996 or better.

9. PROCEDURE

9.1. Sample preparation

Samples such as spice powders, tea leaves and liquid samples are usually adequately homogenous and do not normally require further treatment other than thorough mixing.

Dry and semi-dry goods such as breakfast cereals, biscuits, cakes and boiled sweets should be homogenised using a domestic food processor or Waring-type blender.

Non-dry goods such as dairy products should be homogenised using a domestic food processor / blender or Ultra-Turrax homogeniser.

Note: It may be necessary to slurry certain sample matrices, such as jelly-based confectionery and meat products, with water at a ratio of 50:50 in order to obtain sufficient homogeneity and mobility for SDE analysis. The calculation (9.6) should be adjusted appropriately.

Chewing gum should be hand chopped very finely with a knife.

Small items such as breath mints may be crushed and mixed in a suitable vessel e.g. pestle and mortar.

9.2. SDE

Foods and beverages
Weigh out 0.5-5 g ± 0.05 g of sample depending upon expected BAPs content, using a 2 decimal place balance, into a 100 mL round bottomed flask.

Spices, concentrates and flavourings
Weigh out 0.1-1 g ± 0.01 g of sample depending upon expected BAPs content, using a 2 decimal place balance, into a 100 mL round bottomed flask.

Add 5-6 boiling chips, 50 mL of water and 100 µL of 3-methyl acetophenone internal standard (250 µg/mL).

Spiked samples are prepared at this stage;

10 ug/g spike : Add 40 µL of the 250 µg/mL BAPs mixed standard and 40 µL of the 250 µg/mL menthofuran standard ***per gramme*** of sample using a positive displacement pipette. At least one blank and spike of the appropriate sample must be extracted with each batch.

1 ug/g spike : Add 40 µL of the 25 µg/mL BAPs mixed standard and 40 µL of the 25 µg/mL menthofuran standard ***per gramme*** of sample using a positive displacement pipette. At least one blank and spike of the appropriate sample must be extracted with each batch.

Place 2-3 boiling chips, 2 mL DCM and 100 µL of propyl benzoate internal standard (250 µg/mL) in a 5 mL pear shaped SDE solvent flask.

Connect the sample flask and solvent flask to the appropriate arm of the SDE apparatus securely using PTFE sleeves and joint clips. Add 3 mL DCM to the separation chamber followed by ca. 2 mL water until the water level just begins to flow back into the sample flask. Fit the cold finger condenser securely using a PTFE sleeve. (***Caution: Carry out under a fume hood***).

Note: The apparatus is best supported using a retort stand and clamp, with the heating mantle supported by an adjustable-height lab jack (see Figure 2).

Immerse the solvent flask up to the shoulder in the water bath (70℃) and support the sample flask on the heating mantle. Ensure that the apparatus is upright. Allow the solvent to reflux for 5 minutes then switch on the heating mantle on the highest setting in order to bring the contents to a steady boil.

Allow the sample to reflux for 2 hours then switch off the heating mantle and allow the solvent to continue refluxing for 10 minutes.

Remove the SDE intact from the water bath and allow to cool for at least 5 minutes.

Decant the DCM in the separation chamber into the solvent flask by careful tipping of the apparatus. Take care not to decant any water if possible. Remove the solvent flask and transfer the contents to a 10 mL screw-cap vial.

Transfer an aliquot to a GC autosampler vial and analyse by GC-MS.

Note: Refer to cleaning of glassware.

9.3. GC-MS analysis

Inject 1 μL of sample extract under the conditions given in (6.6).

9.4. Calculation of BAPs levels in samples

Identify the BAPs and internal standard peaks on the basis of their retention time and measure the respective peak areas. Divide the BAP peak area by the propyl benzoate internal standard peak area to obtain the peak area ratio. Calculate the peak area ratios (BAP/PB) for the extracts. From these calculate BAP concentration of the extract from the regression equation of the calibration graph.

Calculate the BAP concentration in the sample from:

$$\text{BAP } (\mu g/g) = \text{BAP concentration in extract } (\mu g/mL) \times \frac{\text{dilution factor*}}{\text{Sample weight (g)}}$$

(* To take account of any sample dilution following slurrying with water (9.1)

10 QUALITY ASSURANCE

In-house reference material spiked at 0.1 and 1 mg/ should be run with every batch. Recovery of spiked samples within the batch should be in the range of 70 – 110%. Any analyte in the blank in-house reference material should be subtracted from the samples.

Reagent blanks should be prepared with each batch using 50 mL water.

Figure 2. SDE apparatus set up

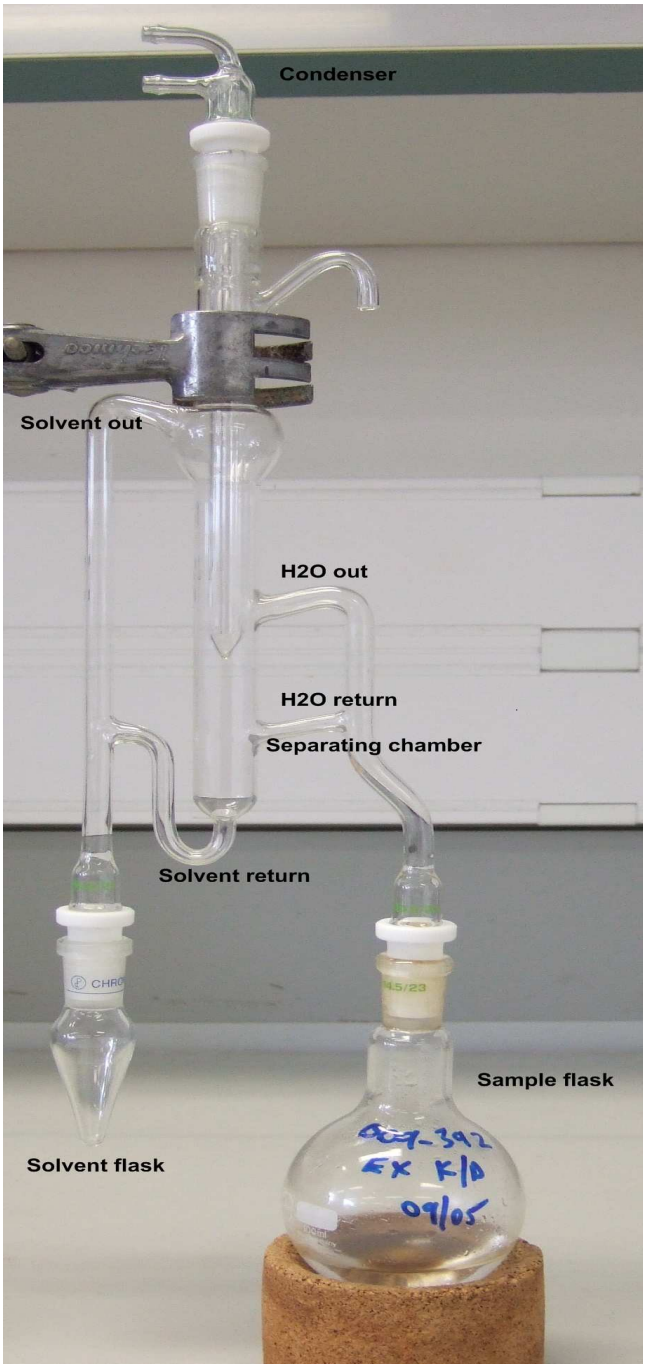

Condenser
Solvent out
H2O out
H2O return
Separating chamber
Solvent return
Sample flask
Solvent flask

Figure 3. GC-MS (TIC) separation of volatile BAPS standards on ZB5-MS column (* Internal standard).

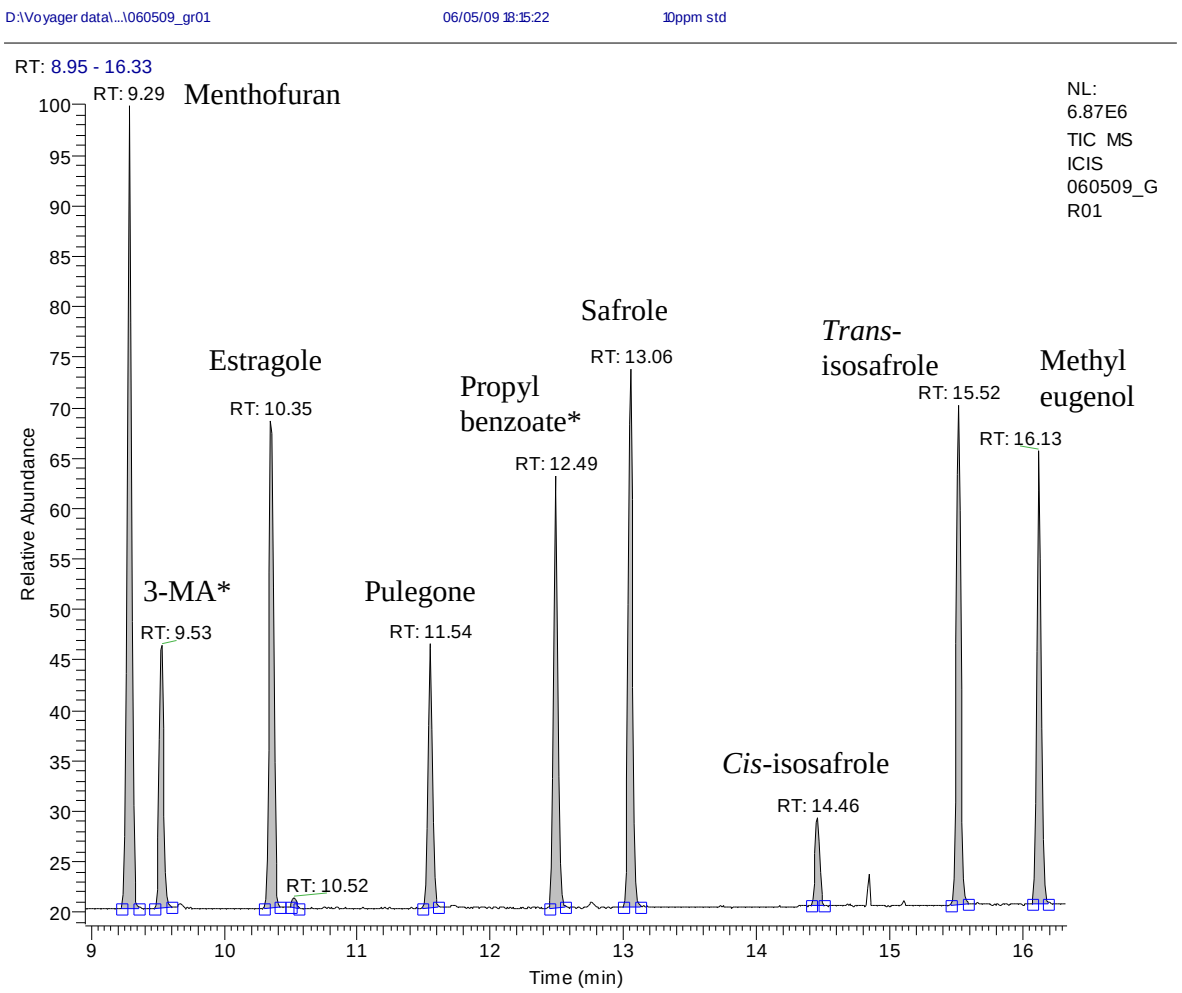


Figure 4. GC-MS (SIM) separation of volatile BAPS standards on ZB5-MS column (* Internal standard).