DEVELOPMENT OF METHODS TO QUANTITATIVELY EXTRACT BIOLOGICALLY ACTIVE PRINCIPLES FROM COMPLEX FOODS, FLAVOURINGS AND HERBS AND SPICES, TO ALLOW THEIR SUBSEQUENT ANALYSIS

FINAL REPORT

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Executive summary

1. This report details the findings of Food Standards Agency Project A01067: Development of methods to quantitatively extract biologically active principles (BAPs) from complex foods, flavourings and herbs and spices, to allow their subsequent analysis. These flavouring compounds cover a range of chemical types and this makes their extraction and determination in foods and beverages analytically challenging.

2. The analytical strategy adopted therefore was to develop three separate methods for each chemical / physical class of BAP:

I. Volatile BAPs by simultaneous distillation-extraction (SDE) of the sample followed by GC-MS quantitation.
II. Coumarin and quassine by solvent extraction of the sample, clean-up using solid phase extraction cartridges and then quantitation by HPLC with UV detection.
III. Hydrogen cyanide by extraction of the sample using acidic medium, enzymatic hydrolysis of glycosidic cyanogens to cyanohydrins, hydrolysis of these to cyanide, and lastly derivatisation using a modified König reaction to form a coloured complex which is determined spectrophotometrically.

3. The methods were validated in single-laboratory tests of a range of different food and beverages. The samples for this validation were selected according to the types of food and beverage likely to contain the BAPs via the addition of herbs and spices and/or flavouring preparations and the maximum levels prescribed in EU

regulations. Eight different matrices (breath fresheners, mint confectionery, yoghurt, canned soup, soft drink, vegetable product, chewing gum and fish product) were validated for five volatile BAPs (menthofuran, estragole, pulegone safrole and methyl eugenol) plus isosafrole. Ten matrices were validated for coumarin and quassine (bakery ware, breakfast cereal, rice pudding, gelatine confectionery, biscuit, sugar confectionery, carbonated soft drink, fruit-flavoured drink, herbal infusion and mixed spice). Three matrices were validated for cyanogenic potential (canned stone fruit, marzipan and alcoholic beverage).

4. The SDE and GC-MS method was subjected to cross validation by a second (independent) laboratory. Samples of breath freshener, chewing gum, fish product, cordial, herbal infusion and mint confectionery were analysed for all five volatile BAPs. The results were mixed. The method is technically challenging and method transfer to the second laboratory was not successful despite a high degree of communication and assistance. Notwithstanding the transferability issue, it is concluded that the use of SDE works well for the extraction and purification / concentration of volatile BAPs from a wide range of food matrices. Sample preparation is the key in providing an adequately dissolved or dispersed matrix that lends itself to SDE analysis. The GC-MS conditions are reasonably straightforward but the GC column needs to display good separation characteristics in order to separate BAPs from other co-extractives.

5. The HPLC method was also subjected to cross validation by the second laboratory. Soft drink, rice pudding, bakery ware, biscuit, breakfast cereal, curry paste and herbal infusion were analysed for coumarin and quassine, and canned

stone fruit, marzipan and alcoholic beverage analysed for cyanogenic potential. There was acceptable agreement in the results for coumarin for all samples except for the herbal infusion. The second laboratory experienced problems with chromatography (split peaks, drifting retention times, interferences) and no conclusions can be drawn on analysis for quassine. It is concluded that coumarin and quassine can be determined in a range of sample types using solvent extraction, clean-up using solid phase extraction cartridges and then quantitation by HPLC with UV detection. The chromatographic conditions are reasonably straightforward but the LC column needs to display good separation characteristics and retention time stability in order to separate coumarin and quassine from other co-extractives and use of a DAD is necessary to avoid false positives.

6. Problems were experienced with transfer of the SOP for the cyanogenic potential method and the second laboratory was not able to produce any results within the time and resource available to them. The HCN method is based on a published procedure and it has been improved further and performed well in the single-laboratory validation. Therefore it is recommended that the SOP should be expanded to give further guidance and improve robustness.

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ANNEX I. Standard Operating Procedure for the simultaneous distillation-extraction GC-MS method for the determination of biologically active principles in foods and beverages

ANNEX II. Standard Operating Procedure for the HPLC method for the determination of coumarin and quassine in foods and beverages

ANNEX III. Standard Operating Procedure for the spectrophotometric method for the determination of the cyanogenic potential of foods and beverages

GLOSSARY

AMG	Amygdalin
AOAC	The Association of Official Analytical Chemists
BAPs	Biologically active principals
COSHH	Control of substances hazardous to health
DCM	Dichloromethane
EFSA	European Food Safety Authority
EU	European Union
FID	Flame ionization detection
FSA	Food Standards Agency
GC	Gas chromatography
HCN	Hydrogen cyanide
HCN_{eq}	Hydrogen cyanide equivalents
HPLC	High performance liquid chromatography
HS	Headspace
IL	Independent laboratory
LC-MS	Liquid chromatography-mass spectrometry
LN	Likens and Nickerson
LOD	Limit of detection
LOQ	Limit of quantitation
3-MA	3-methyl acetophenone
MS	Mass spectrometry
MU	Methyl umbelliferone
PDA	Photodiode array
RSD	Relative standard deviation (%)
SCAN	Full scan mode
SDE	Simultaneous distillation extraction
SFE	Supercritical fluid extraction
SIM	Selective ion monitoring
SOP	Standard operating procedure
SPME	Solid phase micro extraction
TDI	Tolerable daily intake
TIC	Total ion chromatogram
UV	Ultra violet
VIS	Visible

INTRODUCTION

Biologically active principles (BAPs) are undesirable chemical substances that are present naturally in some flavourings and food. In the UK, the Flavourings in Food Regulations (SI 1992 No. 1971) as amended [(1)], control the use of flavourings by laying down concentration limits for certain foodstuffs and beverages. These regulations enact Directives 88/388/EEC [(2)] and 91/71/EEC [(3)] on flavourings for use in foodstuffs. A new EU Regulation [(4)] has been published recently that focuses on the foods and beverages through which BAPs contribute most to the diet. The Regulation sets numerical limits on specific BAPs in food and beverages (Table 1). This new EU Regulation will apply from 20th January 2011.

Reliable analytical methods are required to enforce the legislation and to carry out surveillance for BAPs in food. A FSA-funded critical review of published methods of extraction and analysis for BAPs highlighted a lack of reliable procedures for the quantitative extraction of BAPs from the foodstuffs listed in Table 1 and in particular, complex foods [(5)]. A need was identified to develop quantitative extraction methods, which would be transferable to Public Analyst Laboratories.

The suitability of extraction procedures needs to be judged not only on the efficiency (yield and repeatability) of recovery of the analyte from the complex food matrix but also on the suitability of the extract obtained for the end-determinative step (i.e. at a concentration suitable for analysis and free from any interfering co-extractives). While this project focused mainly on the extraction/clean-up procedures, end determination steps were developed where necessary.

Review of published methodology

Volatile BAPs

Volatile BAPs such as β-asarone, estragole, menthofuran, methyleugenol, pulegone safrole and thujone are relatively small molecules which give both distinct and complex aromas alone and in combination. The available extraction procedures for volatile BAPs cover only a small range of specific foods, beverages and source materials. The available extraction protocols are not adequate to cover the full range of foodstuffs prescribed in the regulations (Table 1). In previous FSA-funded work, studies on safrole and pulegone by gas chromatography-mass spectrometry (GC-MS) revealed that simple solvent extraction or solvent partition from aqueous slurries were not efficient for gum-based mint confectionery and chocolate-based mint flavoured confectionery due to interferences from co-extracted materials such as lecithin and glycerides [(6)]. Similarly, a vapour-phase solid phase micro extraction (SPME) approach met with limited success with poor recovery and repeatability for many analyte/matrix combinations [(7)].

Simultaneous distillation extraction (SDE) based on the microdistillation procedure first described by Likens and Nickerson (LN), is the most commonly used approach for volatile BAPs [(8)]. It is an elegant variation of steam distillation. The SDE technique takes advantage of favourable distribution coefficients of the volatile substances between a solvent and the sample, where the choice of solvent is made not only on the basis of analyte solubility but also to limit potential interferences. The homogenised (often aqueous) sample is introduced into a flask connected to the first arm of the LN apparatus and the (immiscible) solvent is introduced into a

flask connected to the second arm. The two flasks are heated independently and the vapours from both are brought to condense in the central zone of the apparatus which is cooled to ca. 5℃. The aqueous and organic phases separate and flow back into their respective flasks - the organic solvent carrying with it the target analyte, now extracted and isolated from the water slurry of the food. The advantages of SDE, besides the ease of operation, rely on the fact that relatively small volumes of solvent are required compared to sample size; hence both isolation and concentration of analyte(s) is achieved [9]. Bouseta and Collin applied SDE using dichloromethane (DCM) solvent to extract honey for flavour components which included eugenol, thujone and pulegone. They highlighted the critical impact that operating parameters (oxygen level, extraction time and condenser temperature) [10]. Siano et al used SDE with DCM for the extraction of estragole, safrole and methyl eugenol from food products including fresh basil, sauces, sausages and cola-flavoured beverages [11]. Identification and quantitation was achieved using GC-MS with detection limits ranging from 5 to 10 ng/mL. Recoveries ranged between 94 and 105%. Sample sizes were 150-500 g for solid foods and 100-200 mL for liquids. SDE with hexane as solvent was used to extract menthofuran and pulegone from plants [12], safrole from meat products [13] and aroma compounds from cookies and dough models [14].

With regards to alternatives to SDE, supercritical fluid extraction (SFE) has been used to extract BAPs from some food and plant samples [15,16]. SFE has not been demonstrated to be more efficient than direct steam distillation or SDE. SFE requires specialist equipment that is not available in most Public Analyst labs. SPME has been used for pulegone and menthofuran in peppermint [17] but as

mentioned above, the SPME approach met with limited success with poor recovery and repeatability for many analyte-matrix combinations [7].

Quassine

Quassine (also known as quassin) is a crystalline solid that is not suitable for SDE because it is soluble in acetone, methanol and water and it is also insufficiently volatile. This lack of volatility also hampers GC analysis which is reported to give inconsistent results [18]. Immunoassay has been applied to quassine but no validated method is available [19]. Reverse Phase (C_{18}) HPLC with UV detection has been used to determine quassine in spirits by direct injection, with an LOD of 0.1 mg/L and average recovery of ca. 93% [20]. Dou et al. [21] used a phenyl HPLC column with gradient elution for the determination of quassinoids in tissues of *Simaroubaceae* with good response. Either of these methods have the potential to be applicable routinely in an enforcement laboratory, but their extraction protocols require further development to encompass other regulated foodstuffs, particularly bakery wares.

Coumarin

Coumarin is a crystalline solid which is soluble in water, ethanol, ether and chloroform. Celeghini et al showed that hydroalcoholic extraction was suitable for extraction of guaco leaves [22]. While SDE has been used for coumarin extraction, recoveries are reported to be variable. HPLC following solvent extraction is the most sensitive method reported for coumarin and its analogues. He et al., reported a method for the determination of coumarin and its analogues in cassia bark

(cinnamon) using methanol extraction and ultrasonication, followed by reverse-phase HPLC with UV detection [23].

Hydrocyanic acid

Hydrocyanic acid (hydrogen cyanide, HCN) is a colourless liquid with a boiling point of 26℃. There are few satisfactory methods for the determination of hydrocyanic acid in foods. Both free and bound forms (cyanogen e.g. the cyanogenic glucoside amygdalin in apricot kernels) may be found in foods. Enzymic cleavage is often used to release the cyanide from the cyanogen, with detection and quantification based on spectroscopic methods. Methods for the determination of free and bound forms of cyanide in blood based on conversion of bound cyanide into hydrogen cyanide and its subsequent analysis by headspace sampling [24-26] are much more firmly established. Free hydrogen cyanide can be measured directly, or generated from bound cyanides by hydrolysis with acid, typically phosphoric acid. Separation and detection are best based on GC-MS in selected ion monitoring (SIM) mode. Isotopically labelled potassium 13C-cyanide has been used as an internal standard. Headspace-SPME (HS-SPME) can be used in place of static headspace sampling to increase sensitivity [27]. Similar methods have been extended in scope to include body tissues such as blood, liver, kidney, brain, urine, and stomach contents [28].

HCN can also be quantified colorimetrically following derivatization [29]. This method is based on the reaction of HCN with chloramine-T to form chlorocyanide, which in turn produces a red-violet colour in the presence of barbituric acid and pyridine that can be measured spectrophotometrically at 570nm. Other literature methods for the

determination of HCN in foodstuffs are sparse and exhibit disparate efficacies. A colorimetric method based on p-phenylenediamine has been used for canned stone fruit with a reported limit of detection of 0.1 mg/kg but no other validation data could be identified [18]. Other distillation-based extraction techniques are reported to suffer from poor recovery, interference from other sample components and to be time consuming [30-32]. The Association of Official Analytical Chemists (AOAC) alkaline titration method has been used for cassava flour [33] but showed significantly different results to the picrate colorimetric method and the source(s) of error could not be determined. Soup meals have been analysed for HCN using the AOAC method but recovery rates and LODs were not reported [34]. Essers et al. have reported a colorimetric method for the determination of specific cyanogens in cassava products based on the development of a new chromogen that was measured spectrophotometrically at 605nm [35].

Project objectives

The main objective of this project was to provide validated methods for the quantitative extraction of selected BAPs from complex foods prescribed in EU regulations. The methods would be developed primarily with a view to the effective transfer of the necessary available technology and expertise i.e. they would not be unnecessarily complicated or require expensive or unusual equipment, especially since the extraction protocols thus produced may eventually be used by UK enforcement laboratories. The developed methods would also provide the FSA with the means to obtain concentration data from surveys, to allow refined intake estimates of BAPs to be made. The specific scientific objectives were as follows:

1. Development of a method for volatile BAPs based on the quantitative extraction protocols for the extraction and measurement of estragole, coumarin, menthofuran, methyleugenol, pulegone and safrole based on simultaneous distillation-extraction in regulated foodstuffs, using SDE extraction with a view to end-determination using GC-FID and/or GC-MS.

2. Development of a method for quassine based on a quantitative extraction protocol for quassine in bakery wares, based on aqueous extraction and with a view to HPLC end-determination.

3. Development of a method for HCN based either on a quantitative headspace protocol with a GC-FID and/or GC-MS finish or colorimetric procedure in (i) marzipan or nougat, or its substitutes or similar products, and (ii) canned stone fruits.

4. The production of separate SOPs for the above methods sufficient in scope and performance to cover BAP/food commodity combinations where lack of suitable extraction methods have been identified, that are transferable to UK enforcement laboratories, with regard to prescribed limits as laid down in the Regulations.

5. Single laboratory (i.e. in-house) validation of the above SOPs to prove that the methods are efficient and repeatable.

6. External validation of the SOPs by an external (Public Analyst) laboratory to prove that the methods are reproducible and technologically transferable.

The chemical structures of the BAPs and associated chemical species studied are shown in Figure 1.

PART 1: Development of a method for volatile BAPs

The method described by Siano *et al*. [11] was refined for use with the commercially-available Varian-Chrompack SDE apparatus [36]. The method takes advantage of the distribution coefficients of the volatile analytes (BAPS and other volatile components) between the food sample matrix in water and the immiscible solvent DCM. The sample, dissolved or dispersed in water, is introduced into the sample (aqueous) arm of the SDE apparatus while the extracting solvent is placed in a smaller flask on the other arm. The flasks are heated separately and the vapours from each are condensed in a central zone (partition cell) cooled by a cold-finger condenser. In this zone, the aqueous and organic phases separate and flow back into their respective flasks. Figure 2 shows the SDE apparatus set up.

Sample matrices such as spice powders, tea leaves and liquid samples were usually adequately homogenous and did not require further treatment other than thorough mixing.

Dry and semi-dry goods such as breakfast cereals, biscuits, cakes and boiled/jelly sweets were homogenised using a domestic food processor or Waring-type blender. Chewing gum was finely chopped by hand using a knife, then cryo-milled with solid carbon dioxide to leave a finely granulated product.

Non-dry goods were homogenised using a domestic food processor / blender or Ultra-Turrax homogeniser. Some samples such as meat and fish products required dispersion or homogenization in water before sampling.

Once processed, samples were stored at room temperature, chilled or frozen, depending upon their perishability.

For foods and beverages, 0.5-5 g ± 0.05 g of sample was taken depending upon expected BAPS content. The homogenised sample was dissolved or dispersed in 50mL water contained in a 100mL flask along with an internal standard to check distillation efficiency (3-methyl acetophenone). DCM (2mL) was placed in the solvent flask (5mL) to which was added internal standard (propyl benzoate) for quantitative volume adjustment. The partition chamber was charged with DCM (ca. 3mL) and water (ca. 2mL) and a cold-finger condenser fitted and cooled using a circulating chiller set at ca. 5℃.

The solvent flask was heated first by placing in a water bath set at 70℃ and the solvent allowed to reflux for ca. 10 minutes. The sample flask was then brought to a vigorous boil using an electrically heated mantle. The distillation was continued for 2 hours, after which the sample flask heater was switched off and the sample allowed to cool for ca. 20 minutes whilst allowing the DCM solvent to continue refluxing. The SDE apparatus was then removed from the water bath and allowed to cool before the DCM solvent remaining in the partition zone was siphoned into the solvent flask.

The DCM extract was transferred to a 10mL screw-cap glass vial and a small aliquot transferred to a GC autosampler vial (2mL) fitted with a crimp cap for subsequent analysis by GC-MS.

GC-MS conditions

The following conditions were found to be suitable:

Column:	ZB5-MS (5%-phenyl-arylene 95% dimethylpolysiloxane) 30m x 0.25mm x 0.25 um (Phenomenex, UK)
Carrier:	Helium at 1mL/min constant flow
Injector temperature:	250℃
Injection volume:	1μL
Split ratio:	1:50
Oven temperature programme:	
Initial temperature:	80℃
Initial time:	5 min.
Ramp rate (1):	5℃/min.
Final temp (1):	150℃
Ramp rate (2):	100℃/min.
Final temp (2):	325℃
Hold time:	5 min.

MS conditions:

Ionization mode:	Electron impact 70 eV
Detector voltage:	500V
GC interface temp:	280℃
Acquisition modes:	SCAN and SIM

Each BAP was monitored in selected ion mode (SIM) for quantitation on the most abundant ion (shown in bold) along with the masses of appropriate qualifier ions for confirmatory identification:

Menthofuran (m/z **108**, 150,109)

3-Methyl acetophenone (3-MA, m/z **119**, 134) Internal Standard

Estragole (m/z **148**,121, 105)

Pulegone (m/z **152**, 109, 137)

Propyl benzoate (m/z **105**, 123) Internal Standard

Safrole (m/z **162**, 131, 104)

Isosafrole (m/z **162**, 131, 104)

Methyl eugenol (m/z **178**, 163, 103)

SCAN detection mode was used primarily to identify unknown extract components (i.e. other flavour volatiles), especially those eluting close to BAP peaks (peak purity confirmation).

Calibration

Menthofuran is not stable for storage in DCM and was therefore made up separately in cyclohexane. Mixed standards in DCM (n=6) were prepared for GC-MS calibration over the range 0-10 mg/L containing a fixed amount of propyl benzoate internal standard and 3-MA as a distillation efficacy standard, both at 5 mg/L. The analyte peak areas (in SIM mode) were ratioed to the peak area of the propyl benzoate internal standard and plotted against concentration, examples of which are shown in Figure 3. The correlation coefficients for the calibration plots were in the range 0.989 to 0.999, with ranges for the slope and intercept of 0.046 to 0.154 and –0.214 to –0.011 respectively.

Non-BAP flavourings

The following common flavouring compounds were included within the scope of the GC-MS determination for qualitative identification purposes:

Anisole Anethole Carvone Eugenol Isoeugenol

Limonene Menthol Menthone

Small amounts of isosafrole isomers (*cis*- and *trans*-) can co-occur with safrole. Isosafrole can be produced from safrole by isomerisation which usually requires heat and a catalyst. There are currently some minor safety concerns regarding isosafrole so it was included within the scope of this project as well as a possible indicator compound for safrole.

Discussion

The design of the SDE apparatus limits the way in which the heating of the sample and solvent flasks may be arranged. During operation of the SDE apparatus, it was clear that great care must be taken to ensure that the system is clean and leak free. Teflon sleeves were used on all ground glass joints and spring clips used to hold the flasks tightly in place. Fine boiling chips were used in both flasks to ensure smooth refluxing. Close observation of the SDE apparatus on a regular basis was necessary to ensure that the system was working properly, especially the timing of the onset of sample reflux and the relative volumes of the solvents in the partition chamber. Moreover, the distance between the flask necks of the SDE apparatus is only 90mm, which necessitated the use of a small, flexible electrically heated mantle that would allow the sample flask to be heated in close proximity to the solvent flask, which was situated in a water bath. Vigorous boiling of the sample is required but in many cases, very little water distillate was observed dripping into the partition chamber.

During reflux, certain samples were prone to creep up the wall of the sample flask resulting in the deposit of a ring of dry residue above the water line; hence occasional swirling of the sample flask may be necessary during distillation. Certain sample types are prone to frothing which leads to contamination of the SDE apparatus and requires thorough cleaning of the glassware as a result. The use of antifoam solution was thus recommended for such samples. The SDE system therefore requires careful monitoring and 2 hours per sample distillation time.

Apart from the specific matrices discussed below, most sample types did not present any problems during preparation and SDE. However, chewing gum proved very difficult to dissolve or disperse in water due to the presence of gum and wax. Pre-extraction with DCM gave a gummy residue that tended to adhere to the sample flask walls. The use of acid and alkali for digestion of the gum base is discussed below.

Upon homogenisation, the peanuts formed a butter, which upon heating (5g in 50mL of water) frothed up into the SDE apparatus. During the analysis of crisps, some settlement of solid material was observed in the flask even during a vigorous boil, resulting in localised charring of the sample. It was also apparent that samples of fresh herbs (i.e. sage) although being finely chopped, remained largely intact after the 2 hours refluxing, showing no apparent signs of breakdown of the sample matrix. Prior homogenisation of the sample in the water using a Turrax probe was necessary to obtain a finely dispersed sample.

Enzymatic pre-digestion of plant-based and carbohydrate-rich samples was undertaken with a view to improved release of the BAPS during SDE. The enzymes α-amylase and cellulase were added alone and in combination to samples of sage leaves and flavoured crisps, buffered at pH 6 and left at ambient temperature for 24 hours. No visible effects were observed for sage leaves compared to the control sample. There was a clear effect from both enzymes on the crisps sample where the solid mass had separated into a sedimentary layer and a flocculent floating layer containing oil. This suggested that the enzymes had effected a degree of sample digestion.

With a view to using aggressive reagents to break down the sample matrix in order to promote the release of the BAPS, the water was replaced with 1 molar solutions of acetic acid, HCl or KOH for SDE of a standard spike. The BAPS were found to completely degrade in KOH and to partially degrade in HCl, whereas no significant degradation was observed with acetic acid. When the same solvents were applied to the SDE of chewing gum, similar effects were observed and the issue complicated by the appearance of additional peaks in the chromatograms from all three experiments. The results not only show that the use of aggressive reagents may cause significant losses of analytes, but also that the effects of their use are unpredictable.

In a similar experiment, a 25% solution of sodium chloride was used in place of water for the SDE of mint chocolate in an attempt to force a salting-out effect in the sample flask, thereby promoting the release of the BAPS from the matrix. The results showed that there was no significant difference between water and 25% NaCl after 2

hours reflux but after 5 hours in water almost all of the BAPS had degraded or were lost.

All of the analyte peaks from GC-MS analysis were well separated and were identified by their retention time and appropriate response in the total ion (TIC, Figure 4) and in the selective ion monitoring (SIM, Figure 5) mass channels. For all analytes, at least 2 selective SIM ions were available for monitoring sample extracts. Where required, the mass spectra of peaks obtained in sample extracts using scan mode were compared to those found for BAP standards by relating the fragmentation patterns and relative abundances of ions. This was particularly useful for identifying hitherto unknown peaks in chromatograms emanating from non-BAP flavourings and volatiles.

The limit of detection for the GC-MS is approximately 0.1 mg/L, which corresponds to a limit of quantitation for SDE of ca. 0.25 mg/kg for a 5g sample but is dependent upon sample type. This has scope for improvement by utilizing post-SDE concentration using a Kuderna-Danish evaporator (see below).

A ZB-WAX (polyethylene glycol) column (30m x 0.25mm x 0.25 um, Phenomenex, UK) was also used under the same conditions but with a different temperature programme compared to that used for the ZB5 column. This column gave a different elution order compared to ZB5 for the analytes but the separation between 3-methyl acetophenone and propyl benzoate was less efficient but this is not an issue due to the selectivity of the MS detection. However, the use of this column provides a more

polar alternative for confirmation of analyte peaks and/or separation from co-extracted substances if necessary.

Siano et al. [11] have shown that during SDE, estragole, safrole and methyl eugenol were thermally stable for 2 hours at 90℃ after whi ch losses were experienced. Our preliminary experiments with standards spiked into water showed low recoveries (mean 25%) after 1 hour SDE reflux, whereas after 2 hours the recoveries were acceptable. However, there is evidence of a slightly high bias in the lower spike level.

Figure 6 shows the SIM chromatograms obtained for a SDE extract of mint flavour concentrate, where menthofuran, estragole and pulegone have been detected. The presence of the analyte peaks were confirmed by reference to the MS spectra obtained for the corresponding standards. The TIC chromatogram for a SDE extract of flavoured crisps is shown in Figure 7 along with a SDE extract of the same sample spiked with a mixed BAP standard (menthofuran accidentally omitted) and a mixed standard for comparison. Apart from a small number of non-BAP components, only the two internal standards 3-methyl acetophenone and propyl benzoate, were observed in the unspiked crisp sample, whereas all of the analytes were observed in the spiked sample extract at levels and proportions to those in the mixed standard.

In order to determine if a concentration step was feasible for volatile BAPs, the efficacy of a micro Kuderna-Danish concentration was tested with the standard mixture of BAPS used for spiking diluted to 10mL in DCM. The standard was concentrated to a volume of ca. 0.5mL by placing the apparatus in a water bath set

at 100℃. The concentrated extract was then diluted back to 10mL and analysed by GC-MS. The result was compared with that of the original standard mix before concentration. The average recovery of analytes was 105% (range 103 – 107%) showing that there were no significant analyte losses through the concentration process.

Single laboratory validation

Analytical recovery was determined by spiking water (50mL) with a mixed analyte standard (ca. 200 mg/L). Spike volumes of 250uL and 25uL were added to give equivalent sample spike levels of ca. 1 mg/kg and 0.1 mg/kg respectively for a 5g sample weight. Six replicate analyses were carried out at each level and the results are given in Table 2. For the 1 mg/kg (equivalent) spike, the average recovery was 91% (n=6) with a range of between 81% (*trans*-isosafrole) and106% (3-MA distillation recovery internal std). The RSD values were all below 11%. For the 0.1 mg/kg (equivalent) spike, the average recovery was 105% (n=6) with a range of between 95% (methyl eugenol) and111% (estragole). The RSD values were all below 15%.

The following 8 matrices were agreed for the single laboratory validation:

Micro breath fresheners

Chewing gum (mint flavoured)

Mint confectionery (mint imperials)

Dairy product (Natural yoghurt)

Fish product (Flavoured salmon pieces)

Non-alcoholic beverage (ginger cordial)

Canned soup(spiced vegetable)

Processed vegetable product (lemongrass paste)

A summary of the recovery data (n=3) is given in Table 3. Where samples contained natural levels of BAPs, the spike results have been corrected (lemongrass paste 0.9 mg/kg pulegone; Breath fresheners 1.1 mg/kg pulegone; Mint confectionery 8.0 mg/kg pulegone; Chewing gum 6.7 mg/kg pulegone, 9.7 mg/kg menthofuran and 1.0 mg/kg estragole; Processed fish 8.2 mg/kg estragole). Recoveries (n=3) of volatile BAPs from spike food matrices were generally in the range 70-105% with RSDs of 23% or better (most were below 15%) except for menthofuran in yoghurt at 1mg/kg (29.4%).

Given that spiking could only be achieved by adding a mixed BAP solution into the sample flask prior to SDE, it does not mimic the recovery of analytes incurred in the sample matrix. Some problems were encountered during SDE notably the frothing of samples. This was partially ameliorated by the addition of antifoam agent but could not be eradicated in certain samples e.g. chewing gum, canned soup and natural yoghurt, where low recovery of menthofuran (46%) was found for the latter.

Cross validation

The following 6 samples were used for the cross-validation exercise:

Breath freshener (mint) declared contents ‘mint flavouring’

Chewing gum (spearmint) declared contents ‘flavouring’

Fish product (salmon, smoke flavoured) declared contents star anise, cinnamon, fennel, clove, ginger, garlic, black pepper

Cordial (Ginger) declared contents lemongrass, ginger root, no artificial flavours; spiked with menthofuran (2 mg/L), methyleugenol (1 mg/L) and safrole (1mg/L)

Herbal infusion (nettle and fennel as declared contents)

Mint confectionery (imperials) declared contents ‘natural mint flavouring’

The results are given in Table 4 and show reasonable agreement between laboratories in terms of the BAPs identified but some clear differences in concentration between BAPs in several samples. Menthofuran was detected in all but one sample by an independent laboratory (IL), whereas it was detected in only 2 of the 6 samples by Fera, where levels of 33.5 mg/kg and 9.7 mg/kg were reported by the IL and Fera respectively for chewing gum. The IL also reported 22.1 mg/kg menthofuran in breath freshener but it was not detected by Fera. In all of the remaining samples menthofuran was reported at ≤ 1 mg/kg by the IL but not detected by Fera except for herbal tea (0.1 mg/kg). The ginger cordial sample was spiked with menthofuran at a level of 2 mg/L and was detected by the IL at 0.2 mg/L but not by Fera. Menthofuran is known to be unstable, especially to oxidation and degrades significantly when kept in dichloromethane, which is why the menthofuran stock standard was prepared separately from the other standards and in cyclohexane rather than dichloromethane. Recoveries of menthofuran from similar samples spiked at different levels ranged from 57.4-102.9% (Table 3). The IL reported a 50% daily decrease in the slope of the calibration graph for menthofuran over a period of three days. Menthofuran is known to be unstable in DCM and this may account for the higher values observed for the cross-validation samples.

Menthofuran is known to be a minor component of peppermint oil but not spearmint oil.

Estragole was found in all samples by both laboratories except in ginger cordial (IL). Fera reported ca. double the amount found by the IL in breath freshener (29.3 and 13.6 mg/kg respectively) and fish product (8.2 and 4.8 mg/kg), whereas much higher levels were found by Fera in mint confectionery compared to the IL (8.2 and 0.3 mg/kg respectively) and in ginger cordial (123.2 mg/kg and not detected). Estragole is not a recognised component of mint oils but is found in tarragon, basil and pine oil. None of the mint-flavoured products analysed provided any details of the flavourings declared on the list of ingredients. The main flavouring ingredients of the ginger cordial are lemongrass and ginger root, both of which are not known sources of either estragole or any compounds with similar chemical structures to BAPs. Similar levels of estragole were found in chewing gum (ca. 1 mg/kg). The IL reported ca. 47% higher level than Fera in herbal tea but inspection of the mass spectra for the estragole peak in full scan mode by Fera revealed the presence of anethole, a non-BAP isomer of estragole, hence it is possible that this was reported as estragole by the IL. Recoveries of estragole from similar samples spiked at different levels ranged from 68.4-143% (Table 3) with fish product giving the most variable results. The fish product lists fennel and star anise amongst its declared ingredients, which are both sources of estragole but only at low levels, while, anethole is a main component of star anise oil.

Pulegone was detected by both Fera and the IL in ginger cordial (0.7 and 0.2 mg/kg respectively) and in herbal tea (0.8 and 0.9 mg/kg). Pulegone was not detected by

Fera in the fish product whereas the IL reported a level of 1 mg/kg. Large differences in results were observed for breath freshener (1.1 and 20.8 mg/kg), chewing gum (6.7 and 29.3 mg/kg) and mint confectionery (0.8 and 8.2 mg/kg) hence no clear pattern was observed. Recoveries of pulegone from similar samples spiked at different levels ranged from 71.3-123.7% (Table 3). Pulegone occurs naturally in oils from certain species of mint however, none of the mint-flavoured products analysed provided any details of the flavourings declared on the list of ingredients. Chewing gum also proved to be a particularly difficult matrix to analyse (see above), which may be a contributory factor in the variability in results between the two laboratories.

Safrole was not detected in any of the samples by either laboratory apart from a level of 0.2 mg/kg reported by the IL in ginger cordial, which was spiked with safrole at 1 mg/L. A similar situation was observed for non-BAP isosafrole in herbal tea, which was reported as present by both Fera and the IL at levels of 0.4 and 0.3 mg/L respectively but was not spiked into the sample. Recoveries of safrole from similar samples spiked at different levels ranged from 75.0-112.8% (Table 3) with fish product giving the most variable results. Under certain conditions safrole will isomerize to isosafrole hence the presence of the latter may be indicative of safrole.

Methyl eugenol was spiked into the ginger cordial at a level of 1 mg/L and was reported by both Fera and the IL at 0.8 and 0.5 mg/L respectively. Similar levels were found in herbal tea by both laboratories (0.9 and 1.0 mg/kg). The IL reported a level of 0.1 mg/kg in fish product but Fera did not detect any methyl eugenol, and neither laboratory detected methyl eugenol in the remaining samples. Recoveries of

methyl eugenol from similar samples spiked at different levels ranged from 54.2-101.9% (Table 3) with fish product giving the most variable results.

The variability in results between Fera and the IL may be due to several possible factors. Especially, the stability of certain analytes such as menthofuran may be an issue.

The Standard Operating Procedure for the simultaneous distillation-extraction GC-MS method for the determination of biologically active principles in foods and beverages is given in Annex I.

PART 2: Development of a method for quassine and coumarin

Since quassine is not sufficiently volatile for analysis by SDE and coumarin exhibited very poor recovery through the SDE procedure, separate analytical methods for their analyses were required. Both quassine and coumarin may be extracted into aqueous alcohol and reverse-phase HPLC [20-23], so a single method was developed with a view to encompassing both coumarin and quassine. Moreover, an unpublished outline method for coumarin was made available by the General Directorate for Competition Policy, Consumer Affairs and Fraud Control [37]. This method was used as the basis for development.

Briefly, quassine and coumarin were extracted from foods and beverages using 90% aqueous methanol by blending or sonication/shaking. For foods and beverages, 2-5 g ± 0.05 g of sample was taken depending upon expected analytes content. Following centrifugation, an aliquot of the supernatant was cleaned up using dispersive solid phase extraction (SPE). The extract was diluted with water at a volume ratio of 1:3 (sample extract:water) and loaded on to a primed C_{18} SPE cartridge. Unwanted coextractives were removed by washing with water followed by 20% methanol. The quassine and coumarin (and internal standard methylumbelliferone, MU) were selectively eluted with 60% methanol, filtered (0.2 um) and analysed by HPLC.

A mixed quassia standard containing about 13% of quassine and ca. 40% of neoquassin was obtained from Trifolio-M GmbH, Germany. Quassine and coumarin were readily separated from other cinnamon components (cinnamic acid and cinnamaldehyde) and from the internal standard using gradient elution. The quassine

standard comprises three components, two of which co-elute under the developed HPLC conditions:

Injection volume:	20µL
Mobile phase:	Mobile phase A – Acetonitrile
	Mobile phase B – Water, 0.5% Acetic acid
Elution:	Gradient at 1 mL/min
Column:	Zorbax C_8 5 µm 250 x 4.6 mm (other columns may be suitable)
Detector:	Photodiode array (PDA)
	Monitoring wavelength quassine/MU: 256 x 4 nm
	Monitoring wavelength coumarin/MU: 280 x 4 nm
PDA conditions:	Reference wavelength: 600 x 4 nm
	Spectrum range: 190-400 nm
Peak retention times:	Methyl umbelliferone *ca.* 10.7 mins
	Coumarin *ca.* 12.5 mins
	Quassine *ca*. 13.4 and 14.1 mins

Calibration

The regression equation was determined from analyte/internal standard peak area ratios with a correlation coefficient of 0.996 or better. The limit of detection for both substances is less than ca. 1 mg/L but this is matrix dependent.

Excellent correlation was achieved over the calibration range 0.25 – 260 mg/L for coumarin and 0 – 18 mg/L for quassine (Figure 8). Figure 9 shows a similar

correlation for coumarin spiked into caramel biscuit over the concentration range 5 - 140 mg/kg.

The HPLC-PDA chromatogram for a standard mix of coumarin and quassine at 10 mg/L is shown in Figure 10 and reveals good separation between the analytes and the MU internal standard, along with the HPLC-PDA chromatogram obtained from an extract of cinnamon powder spiked with quassine. Very good separation between analytes and internal standard was achieved.

Single laboratory validation

The following 10 sample matrices were validated:

Bagel	Breakfast cereal
Biscuit	Gelatine confectionery
Rice pudding	Sugar confectionery
Mixed spice	Herbal infusion
Tonic water	Fruit-flavoured soft drink

The tonic water and a fruit-flavoured soft drink were included with a view to their use as test materials for the cross-lab validation study. Since it was difficult to obtain a proprietary mixed spice sample for spiking that did not contain cinnamon or nutmeg, the test sample was prepared in-house by mixing equal amounts of dried ground coriander, cumin, ginger and turmeric. The gelatine confectionery (jelly babies) was homogenised by dissolving in hot water, evaporating the excess water slowly while stirring and allowing to cool and set.

The relatively high fat content (ca 20%) of the biscuit sample, necessitated a precipitation step prior to HPLC analysis that was achieved by freezing of the methanolic extract at ca. –18℃ followed by centrif ugation.

Samples were extracted according to the procedures given in the SOP (Annex II). Samples were analysed by direct analysis (no SPE step) and by analysis using an optional SPE clean up step (except for the tonic water and the fruit-flavoured soft drink, which were analysed directly only). The herbal infusion (camomile tea) was analysed as dry leaves.

All samples were spiked at levels of 2 and 20 mg/kg for both coumarin and quassine, except for the mixed spice, which was spiked at 200 and 2000 mg/kg. The mean recovery figures (n=4) are given in Tables 5 and 6.

Direct analysis

The range of average recoveries for coumarin at the 2 mg/kg spike level was 97-108% except for the (‘plain’) bagel sample (170%) and the camomile infusion, which contained interfering peaks that masked both the internal standard and the coumarin peak at both spike levels. Examination of the peak spectra revealed no spectra attributable to coumarin or quassine. This was observed in both the extracts of dried leaves and in the prepared infusion samples.

The chromatogram for the extract of the bagel sample also showed evidence for an interfering peak. However, the recovery improved for the same extract after SPE clean up at 95%. All of the RSD values were < 13% indicating good precision except

for the bagel sample (18.6%). At the 20 mg/kg spike level, the average recovery range was very good a 98-110% with all RSD values < 3%. The recovery of coumarin (and quassine) from the mixed spice sample was also good at 107-116% with all RSD values ≤ 7.2%.

While it is clear that quassine is only likely to be present in soft drinks and fine bakery, all samples were spiked with quassine to test the scope of the method. The range of average recoveries at the 2 mg/kg spike level was 95-110 except for gelatine confectionery (121%) and biscuit (135%) indicating the presence of interfering substances in these matrices. All RSD values were < 10% except for tonic water (16.2%). At the 20 mg/kg spike level, the recoveries were over the range 93-111% with all RSD values below 5% except for breakfast cereal (15%). As for coumarin, the camomile infusion extract contained interfering peaks that masked quassine peaks at both spike levels. Examination of the peak spectra revealed no characteristics clearly attributable to quassine (Figure 11).

Analysis with SPE cleanup

Coumarin recoveries at the 2 mg/kg level were in the range 92-109% with RSD values all < 8%. The recovery for the bagel extract improved from 170% to 95% after SPE clean up. The recoveries at the 20 mg/kg spike level were also good at 90-104% with RSD values all < 4%. The recoveries for the spice sample spiked at 200 and 2000 mg/kg showed similar recoveries and RSD values to the directly analysed extract. SPE cleanup of the camomile infusion sample was not successful at removing the interfering peaks hence coumarin recovery could not be determined.

Quassine recoveries at the 2 and 20 mg/kg spike levels were in the range 104-130% and 93-125% respectively. All RSD values were ≤ 11% for both spike levels except for breakfast cereal (19.6%) and rice pudding (22.1%) spiked at the 20 mg/kg level. The higher recoveries are thought to be due to losses of internal standard. SPE cleanup of the camomile infusion sample was not successful at removing the interfering peaks hence quassine recovery could not be ascertained. Recoveries of quassine from the spice sample spiked at 200 and 2000 mg/kg showed good recoveries and RSD values both by direct analysis and by analysis with SPE cleanup.

To further test the scope of the SPE cleanup, repeat analysis of a range of foodstuffs with cinnamon as a declared ingredient or flavouring was undertaken. The results are given in Table 7 and demonstrate very good repeatability (n=8) over a coumarin content range of between 10 and 71 mg/kg, and at ca, 2,500 mg/kg in cinnamon powders. The calculated RSD values were in the range 1 – 6%. In a similar experiment, cinnamon powder and caramel biscuit spiked with quassine at 3717 and 74 mg/kg respectively and analysed repeatedly (n=8) gave %RSD values of 14 and 9 respectively.

Cross validation

The cross-validation of the method for the determination of coumarin and quassine using the developed method was carried out by the IL on soft drink, rice pudding, cinnamon bagel, breakfast cereal, curry paste and herbal infusion. Some of the matrices required fortification as shown in Table 8, where the results obtained by Fera and the IL are summarised.

There is reasonably good agreement between Fera and the IL in the levels of coumarin determined in all of the matrices except the herbal infusion (see below). Conversely, agreement in the levels of quassine determined in all of the matrices was less comparable. False negative results were reported for the soft drink, cinnamon bagel and cinnamon biscuit samples, and a false positive result reported for rice pudding. The results for the breakfast cereal, curry paste and herbal infusion were in agreement i.e. zero or not determined.

A relatively high level of coumarin (96 mg/kg) was reported by the IL for the herbal infusion compared to the Fera result (zero). The herbal infusion contained a high number of peaks (suspected to be due to polyphenols) that can co-elute with both coumarin and quassine. The HPLC separation achieved by the IL was clearly sub-optimal since they reported that while both C_{18} and C_8 columns were tried, only the C_8 seemed to work. Moreover, the quassine peak was observed as at least a doublet. The peaks also eluted quite close together and the retention times were not stable. The IL commented that a longer stabilisation time might be necessary but such large drifts in retention time were not observed at, so it is likely that better column temperature control is required.

The Standard Operating Procedure for the HPLC method for the determination of coumarin and quassine in foods and beverages is given in Annex II.

PART 3: Development of a method for HCN

Preliminary work on a headspace method for HCN revealed very poor sensitivity due to adsorption of the HCN onto instrument surfaces. Subsequent development could not improve the situation so after consultation with the FSA, it was agreed that this approach should be abandoned in favour of the colorimetric procedure.

The methodology selected for development was based on that described by Essers et al for the colorimetric determination of total cyanogenic potential in cassava products [(35)]. Briefly, the sample is extracted into acidic medium, buffered and the glycosidic cyanogens hydrolysed enzymatically to cyanohydrins. The cyanohydrins are then chemically hydrolysed under alkaline conditions to cyanide, which undergoes complexation with Chloramine-T, dimethylbarbituric acid and isonicotinic acid in a modified König reaction. The resultant complex is determined spectrophotometrically at 605nm and the results expressed as HCN equivalents (HCNeq). Quantification was achieved by external standardization using amygdalin (Figure 12), which is the major precursor cyanogenic glucoside found in stone-fruit kernels.

The method was refined and optimised for use with food and beverage samples. Sample extraction was carried out under chilled conditions in order to minimise losses of free HCN and the colour formation time optimised at 30 minutes (Figure 14). While extraction of cyanogenic glucoside from the alcoholic beverage was facile, the canned stone fruit and marzipan matrices required longer extraction times (2 hours) and a second extraction of 30 minutes with pooling of the extractants. A third extraction of 30 minutes did not improve recoveries significantly.

Relatively high levels of soluble protein and/or carbohydrate were considered to be responsible for the production of hazy/turbid marzipan extract supernatants that could not be clarified by centrifugation. This was ameliorated to some extent by taking a small sample weight (1g) and by filtration of the supernatant through a 0.45um syringe filter prior to hydrolysis.

Calibration

The method was calibrated primarily using standard solutions of potassium cyanide expressed as HCNeq with excellent linearity (R^2 > 0.999). Similar results were observed for calibration with amygdalin standard (following the hydrolysis procedure) but with a 15% reduced response characterised by the slope of the calibration line (Figure 13). The observed difference in response is considered to be due largely to the purity of the amygdalin standard used (Sigma-Aldrich No. A6005 from apricot kernels with a stated purity of 99%). However, the purity is stated on an anhydrous basis since amygdalin occurs largely as the trihydrate analogue, which is in line with the stated water content of ca. 11%. Amygdalin trihydrate has a molecular mass of 511.49 compared to anhydrous amygdalin (457.29). Thus the mole ratio of HCN (27.03) to amygdalin trihydrate is 27.03/511.49 = 0.05285. This ratio has been used throughout the method development and validation analyses. Note: The mole ratio given in the EFSA opinion is 0.0591 but this is based on anhydrous amygdalin [(38)].

Single laboratory validation

The method was validated against the following 3 sample matrices:

- Alcoholic beverage (liqueur) at 5 and 50 mg/L HCN equivalents

- Marzipan (60% almond content) at 5 and 35 mg/kg HCN equivalents
- Canned stone fruit (cherries in syrup) at 1 and 5 mg/kg HCN equivalents

Samples (n=6) were spiked with a solution of amygdalin in water immediately prior to extraction along with control samples. The results are summarized in Table 9 and have been corrected for background (control) HCNeq content. The amygdalin calibration showed good reproducibility over the range 0 – 18 mg/kg HCNeq with R^2 values of 0.99 and higher. Amygdalin standards in water were found to be stable at ambient temperature as were the other reagents except for chloramine-T solution, which required fresh preparation each day.

Recoveries of amygdalin spiked into the alcoholic beverage (5mL) close to the prescribed maximum level (35 mg/kg HCN) showed a very good mean recovery of 99.2% (n=6) with an RSD value of 0.8%. At the lower spike value the mean recovery of 117% (RSD = 3.2%).

Amygdalin spiked into the canned cherries sample (5g) close to the prescribed maximum level (5 mg/kg HCN) and at ca. 1 mg/kg also showed very good mean recoveries of 95.2 and 98.9% respectively. The variability in results was larger in comparison to the alcoholic beverage spiked samples as revealed by the RSD values of 12.7% and 23.9% respectively. This is considered to be due largely to sample inhomogeneity.

Due to the problems associated with extraction discussed above, the spiking of the marzipan was carried out on 1g samples. Both the high spike (close to the

prescribed maximum limit of 50 mg/kg HCN) and the low spike (ca. 5 mg/kg) showed lower mean recoveries of 65.5% and 59.9% respectively. The RSD values were very similar for both spike levels (13.7% and 13.9%). Increasing the enzyme hydrolysis time and volume did not improve recovery.

The marzipan sample showed the highest mean background level of 5.8 mg/kg HCNeq (RSD 3.8%) equivalent to 11.6% of the prescribed maximum limit. The canned cherries showed the highest background level with respect to the prescribed maximum limit at 46.0%, whereas the corresponding figure for the alcoholic beverage was 0.6%.

Cross validation

Three samples were prepared for cross validation; the marzipan and stone fruit samples unspiked and the alcoholic beverage (liqueur) spiked with amygdalin to give a concentration of HCN equivalents of 5 mg/L.

A number of points of clarity in the SOP were indicated by the IL that required several iterations of the prescriptive text prior to analysis of the samples. The IL reported that cyanide calibration gave the expected linear response and sensitivity but calibration with amygdalin standards, while sufficiently sensitive was exponential rather than linear over the prescribed concentration range. When attempts were made to analyse the samples, poor colour development was observed despite repeated preparation of reagents, especially the buffer solutions and colour reagent. At Fera, the colour reagent was stable for at least 2 months and since the chloramine-T is prepared daily, the only other likely sources of error are the buffers

(accuracy of pH) and/or the b-glucosidase activity. No results for the cross-validation study were returned therefore a comparison with Fera results could not be made.

Discussion

It is not clear why the method could not be applied successfully at the IL except that it may generally lack robustness. The IL used plastic vials in place of glass, which gave acceptable calibration with the KCN standards but not with the amygdalin calibration, which was manifested as poor colour development. This could also be attributable to activity of the enzyme but Fera observed no such problems, so there is some concern regarding the enzyme stability. Greater detail on the preparation and checking of the enzyme may therefore be critical to the success of the method, then more precise directions should be given for preparation and storage.

The Standard Operating Procedure for the spectrophotometric method for the determination of the cyanogenic potential of foods and beverages is given in Annex III.

Conclusions and recommendations

All of the initial and revised milestones and deliverables agreed for this project have been achieved. The method development, single-laboratory validation and SOP production objectives have been achieved but it has not been possible to demonstrate that the methods for volatile BAPs, quassine and cyanogenic potential are sufficiently robust for the analysis of incurred samples, and that they can be used by other laboratories. Extensive development and adaptation was undertaken on the method for the extraction of volatile BAPs by SDE with GC-MS detection in order to broaden the analytical scope. The HPLC method for coumarin was refined to encompass quassine and to broaden the analytical scope, and an optional SPE cleanup step developed. The spectrophotometric method for total cyanogenic potential was developed and optimized for the analysis of three food/beverage matrices.

Over the course of the project, seven progress reports have been issued and a Standard Operating Procedure for each method has been produced, fulfilling the agreed project deliverables.

SDE

- The use of SDE for the extraction and purification/concentration of volatile BAPs has been shown to work in principle for a wide range of food matrices. Sample preparation has shown to be key to the provision of an adequately dissolved or dispersed matrix that will lend itself to SDE analysis. The presence of small amounts of alcohol as found in cocktail drinks, does not

appear to affect the SDE partition between water and DCM. Conversely, the extraction of chewing gum and fish product has been problematic.

- Cleaning and set up of the SDE apparatus and the conditions of use require close attention to detail, which must be given adequate provision in a Standard Operating Procedure.
- The GC-MS conditions are reasonably straightforward but the GC column needs to display good separation characteristics in order to separate BAPs from other co-extractives. SIM/TIC and SCAN detection modes should be used where appropriate to not only confirm the presence of BAPs but also to ensure that co-extracted volatile substances, which may be present in much larger amounts, are not misidentified as BAPs.

Coumarin/quassine

- In terms of coumarin extraction efficacy and precision, the method appears generally to be fit for purpose except for the camomile infusion sample where the presence of HPLC peaks due to co-extracted compounds (polyphenols?) gave rise to significant interference at the spike levels added.
- Those sample types most likely to contain quassine i.e. fine bakery wares (bagel) and soft drink both showed acceptable recoveries and precision.
- It is recommended that the SPE clean up should be used to remove co extracted material only where necessary for HPLC analysis as it effectively doubles the analysis time and small losses of internal standard may occur.
- The cross-validation results show that the method works well for the determination of coumarin in all matrices tested except for the herbal infusion, where the presence of polyphenols is suspected to interfere.

- The method is less successful for quassine which has a weak chromophore, especially for the low levels present in the samples and spikes, which reflect the maximum limits in the regulations. This suggests that further development of the cleanup/concentration stage is required to remove such interferences, and/or an alternative detection method to UV absorption is used which provides greater selectivity e.g. LC-MS.
- It is clear that the chromatographic conditions must be stabilised for acceptable, repeatable separation of the analytes and the internal standard, and that the use of photodiode-array detection is desirable for analyte confirmation.
- The amount of internal standard used needs to be reviewed. It was not possible to carry this out during the project due to time and financial pressures.

HCN

- The method was developed and successfully single-laboratory validated for canned stone fruit, marzipan and alcoholic beverage, at concentrations at the prescribed maximum level and below.
- Calibration using both KCN and amygdalin was successful
- Problems with insoluble matter were experienced with analysis of marzipan.
- The cross validation of the method was unsuccessful. The reasons for this are not clear hence identification of the factors contributing to the lack of method robustness need to be identified. For example, whether or not plastic vials can be used and the use of enzymes to predigest the proteins and carbohydrates

in marzipan should be investigated.It was not possible to carry out these extra experiments due to contractual time and finance pressures.

REFERENCES

1. The Flavourings in Food Regulations 1992. Statutory Instrument 1992 No. 1971.
2. Council Directive 88/388/EEC of 22 June 1988 on the approximation of the laws of the Member States relating to flavourings for use in foodstuffs and to source materials for their production.
3. Commission Directive 91/71/EEC of 16 January 1991 completing Council Directive 88/388/EEC on the approximation of the laws of the Member States relating to flavourings for use in foodstuffs and to source materials for their production.
4. Regulation (EC) No 1334/2008 of The European Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC
5. Turner, J.S., and Shuker, D.E.G., Review of methods of analysis for biologically active principles. FSA Project Report for A01041.
6. Johnston, S. D., Hird, S. J., Brown, N. A. and Moffat, C. F., CSL report 1994 FD94/138 Biologically active principles in herbal teas, mint teas and mint products.
7. Development and validation of a solid phase microextraction method for the determination of coumarin and safrole in foods. Project A01008. Campden & Chorleywood Food Research Association. FSA Working Party on Food Additives, Annual review of current research projects, 2000. Published at http://archive.food.gov.uk/pdf_files/food_additives.pdf and updated in the

Programme Review document at http://www.food.gov.uk/multimedia/pdfs/a01anda02review

8. Likens, S., and Nickerson, G., 1964, Determination of certain hop oil constituents in brewing products. *ASBC Proceedings*, 5-13.
9. Godefroot, M., et al., 1981, New method for quantitative essential oil analysis. *Journal of Chromatography*, **203**, 325-335.
10. Bouseta, A., and Collin, S., 1995, Optimized Likens-Nickerson methodology for quantifying honey flavours. *Journal of Agricultural and Food Chemistry*, **43**, 1890-1897.
11. Siano, F., et al., 2003, Determination of estragole, safrole and eugenol methyl ether in food products. *Food Chemistry*, **81**, 469-475.
12. Orav, A., Kahn, J., 2001, Determination of peppermint and orange aroma compounds in foods and beverages. *Proceedings of the Estonian Academy of Sciences and Chemistry*, **50**, 217-225.
13. Tateo, F., et al., Determination of safrole and isosafrole in meat based products. *Industrie Alimentari*, **38**, 941-945.
14. Prost, C., et al., 1993, Extraction of cookie aroma compounds from aqueous and dough model system. *Journal of Food Science*, **58**, 586-588.
15. Ehlers, D., Hilmer, S., and Bartholomae, S., (1995), HPLC analysis of supercritical carbon dioxide extracts of cinnamon and cassia in comparison with cinnamon and cassia oils. *Zeitschrift für Lebensmittel Untersuchung und-Forschung*, **200**, 282-288.

16. Aghel, N; Yamini, Y; Hadjiakhoondi, A and Pourmortazavi SM , (2004), Supercritical carbon dioxide extraction of *Mentha pulegium* L. essential oil. *Talanta*, 62, 407-411.
17. Rohloff, J., 1999, Monoterpene composition of essential oil from peppermint (*Mentha x piperita* L.) with regard to leaf position using solid phase microextraction and gas chromatography/mass spectrometry analysis. *Journal of Agricultural and Food Chemistry*, **47**, 3782-3786.
18. The investigation of methods for the determination of active flavouring principles in food and drink. Report prepared at the Laboratory of the Government Chemist for the Food Science Division of the Ministry of Agriculture, Fisheries and Food. 1983.
19. Robbins, R., et al., 1984, An enzyme-linked immunosorbent assay for quassine and closely related metabolites. *Analytical Biochemistry*, **136**, 145-156.
20. Lander, V., et al., 1990, use of solid-phase extraction for rapid sample preparation in the determination of food constituents. 2. asarone, quinine, coumarin and quassine in spirits. *Zeitschrift fur Lebensmittel-Untersuchung und-Forschung*, **190**, 410-413.
21. Dou, J., et al., 1996, Qualitative and quantitative high performance liquid chromatographic analysis of quassinoids in Simaroubaceae plants. *Phytochemical Analysis*, **7**, 192-200.
22. Celeghini, R., Vilegas, J., Lancas, F., 2001, Extraction and quantitative HPLC analysis of coumarin in hydroalcoholic extracts of *Mikania glomerata* ('guaco') leaves. *Journal of the Brazilian Chemical Society*, **12**.

23. He, Z-D et al., 2005, Authentication and quantitative analysis on the chemical profile of cassia bark (*Cortex cinnamomi*) by high-pressure liquid chromatography. *Journal of Agricultural and Food Chemistry*, **53**, 2424-2428.
24. Calafat, A.M. and Stanfill, S.B. (2002)Rapid quantitation of cyanide in whole blood by automated headspace gas chromatography. *Journal of Chromatography B*. **772** (1) 131-137.
25. Dumas, P., Gingras, G. and LeBlanc, A. (2005) Isotope dilution-mass spectrometry determination of blood cyanide by headspace gas chromatography. *Journal of Analytical Toxicology* **29** (1) 71-75.
26. Murphy, K.E., Schantz, M.M., Butler, T.A., Benner, B.A., Wood, L.J. and Turk, G.C. (2006) Determination of cyanide in blood by isotope-dilution gas chromatography-mass spectrometry. *Clinical Chemistry* **52** (3) 458-467.
27. Frison, G., Zancanaro, F., Favretto, D. and Ferrara, S.D. (2006) An improved method for cyanide determination in blood using solid-phase microextraction and gas chromatography/mass spectrometry. *Rapid Communications in Mass Spectrometry* **20** (19) 2932-2938.
28. Zhang, C.S., Zheng, H., Jin, O.Y., Feng, S.Z. and Taes, Y.E.C. (2005) Cyanide distribution in human tissue, determined by GC/ECD/HS. *Analytical Letters* **38** (2) 247-256.
29. Brereton, P., et al., 2003, Analytical methods for the determination of spirit drinks. *TrAC Trends in Analytical Chemistry*, **22**, 19-25.
30. Wiliams, H., Edwards, T., 1980, Estimation of cyanide with alkaline picrate. *Journal of the Science of Food and Agriculture*, **31**, 15-22.

31. Srinivisan, E., Krishnamoorthy, A., Anbumani, S., 1994, detection of hydrocyanic acid (HCN) in green fodder. *Indian Journal of Animal Sciences*, **64**, 1303-1304.

32. Ayres, J., et al., 2001, A rapid semi-quantitative procedure for screening hydrocyanic acid in white clover (*Trifolium repens* L.). *Australian Journal of Experimental Agriculture*, **41**, 515-521.

33. Ukhun, M., Nkwocha, F., 1989, The hydrocyanic acid (HCN) content of garri flour made from cassava (Manihot spp.) and the influence of length of fermentation and location of source. *Food Chemistry*, **33**, 107-113.

34. Akpanabiatu, M., et al., 1998, Evaluation of some minerals and toxicants in some Nigerian soup meals. *Journal of Food Composition and Analysis*, **11**, 292-297.

35. Essers, A., Bosveld, M., van der Grift, R., and Voragen, A., (1993), Studies on the quantitation of specific cyanogens in cassava products and introduction of a new chromogen. *Journal of the Science of Food and Agriculture*, **63**, 287-296.

36. Chrompack, (1988), Users manual for micro-steam distillation extraction apparatus. Chrompack International, Middleberg, The Netherlands.

37. General Directorate for Competition Policy, Consumer Affairs and Fraud Control. Found at: http://www.dgccrf.bercy.gouv.fr/anglais.htm, August 2009.

38. EFSA Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) on hydrocyanic acid in flavourings and other food ingredients with flavouring properties. The EFSA Journal (2004), 105: 1-28.

TABLES

Table 1. Numerical limits for BAPs in foods and beverages [4]

BAP	Foodstuff	Limit (mg/kg)
β-Asarone	Alcoholic beverages	1
Estragole* (1-allyl-4-methoxybenzene)	Dairy products	50
	Processed fruit, vegetables (incl. mushrooms, fungi, roots, tubers, pulses and legumes), nuts and seeds	50
	Fish products	50
	Non-alcoholic beverages	10
Hydrocyanic acid	Nougat, marzipan, or its substitutes or similar products	50
	Canned stone fruits	5
	Alcoholic beverages	35
Menthofuran	Mint/peppermint containing confectionery except micro breath-freshening confectionery	500
	Micro breath-freshening confectionery	3000
	Chewing gum	1000
	Mint/peppermint containing alcoholic beverages	200
Methyleugenol* (4-allyl-1,2-dimethoxy -benzene)	Dairy products	20
	Meat preparations and meat products, including poultry and game	15
	Fish preparations and fish products	10
	Soups and sauces	60
	Ready-to-eat savouries	20
	Non-alcoholic beverages	1
Pulegone	Mint/peppermint containing confectionery except micro breath-freshening confectionery	250
	Micro breath-freshening confectionery	2000
	Chewing gum	350
	Mint/peppermint containing non-alcoholic beverages	20
	Mint/peppermint containing alcoholic beverages	100
Quassin	Non-alcoholic beverages	0.5
	Bakery wares	1
	Alcoholic beverages	1.5
Safrole* (1-allyl-3,4-methylene -dioxy benzene)	Meat preparations and meat products, including poultry and game	15
	Fish preparations and fish products	15
	Soups and sauces	25
	Non-alcoholic beverages	1
Teucrin-A	Bitter-tasting spirit drinks or bitter[1] Liqueurs[2] with a bitter taste Other alcoholic beverages	5 5 2
Thujone(α- and β-)	Alcoholic beverages, except those produced from *Artemisia* species	10
	Alcoholic beverages produced from *Artemisia* species Non-alcoholic beverages products from Artemisia species	35 0.5
Coumarin	Traditional and/or seasonal bakery ware containing a reference to cinnamon in the labelling	50
	Breakfast cereals including muesli	20
	Fine bakery ware, with the exception of traditional and/or seasonal bakery ware containing a reference to cinnamon in the labelling	15
	Desserts	5

[(*) The maximum levels shall not apply where a compound food contains no added flavourings and the only food ingredients with favouring properties which have been added are fresh, dried or frozen herbs and spices. After consultation with the Member States and the Authority, based on data made available by Member States and on the newest scientific information,

and taking into account the use of herbs and spices and natural flavouring preparations, the Commission, if appropriate, proposes amendments to this derogation.

(1) As defined in Annex II, Paragraph 30 of Regulation (EC) No 110/2008

(2) As defined in Annex II, Paragraph 32 of Regulation (EC) No 110/2008]

Table 2. SDE recovery of BAPs from 50mL of water containing 1 mg/L and 0.1 mg/L.

Analyte	**Replicate (% recovery)**								
1mg/L	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**STD DEV**	**RSD (%)**
Menthofuran	86	93	96	103	98	97	96	5.59	5.9
Estragole	91	92	90	95	97	90	92	3.11	3.4
Pulegone	97	93	82	85	90	85	89	5.77	6.5
3-MA	100	97	109	112	106	113	106	6.58	6.2
Safrole	92	100	88	91	95	88	92	4.76	5.1
Cis-Isosafrole	87	88	82	87	88	79	85	3.91	4.6
Trans-isosafrole	87	86	76	83	85	69	81	6.88	8.5
Methyl eugenol	99	92	78	80	90	76	86	9.15	10.7
0.1 mg/L	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**STD DEV**	**RSD (%)**
Menthofuran	104	120	113	88	115	75	103	14.09	13.7
Estragole	103	101	111	111	134	108	111	5.23	4.7
Pulegone	105	85	103	104	128	103	105	9.33	8.9
3-MA	113	85	104	122	116	97	106	15.57	14.7
Safrole	87	110	106	104	132	115	109	10.24	9.4
Cis-Isosafrole	95	95	97	90	133	103	102	2.98	2.9
Trans-isosafrole	93	112	104	104	136	112	110	7.27	7.0
Methyl eugenol	82	95	88	90	114	100	95	5.69	6.0

Table 3. Single laboratory validation data for SDE method (% recovery)

Matrix	Spike level (mg/kg)	Menthofuran	Estragole	Pulegone	Safrole	*Cis*-isosafrole*	*Trans*-isosafrole*	Methyl eugenol	3-MA**
Breath fresh.	100								
Mean		87.4	76.6	94.3	86.2	84.3	84.6	80.4	90.3
RSD(%)		6.7	5.9	9.7	18.8	23.6	21.9	20.2	9.1
Breath fresh.	1000								
Mean		70.5	68.4	76.6	97.2	99.2	98.3	93.3	89.5
RSD(%)		10.8	9.0	10.8	6.2	7.4	9.4	22.3	4.1
Mint confec.	25								
Mean		81.7	89.0	123.7	85.7	84.8	86.1	85.2	94.6
RSD(%)		1.2	14.4	13.8	11.9	11.8	13.2	14.7	12.4
Mint confec.	250								
Mean		83.1	89.2	89.7	99.5	95.4	98.6	95.2	97.1
RSD(%)		22.1	6.8	9.7	11.2	12.4	12.2	9.1	7.0
Yoghurt	1								
Mean		46.3	78.3	73.5	80.6	70.1	74.0	72.9	95.9
RSD(%)		29.4	5.2	5.2	2.5	1.8	2.6	8.5	5.1
Yoghurt	10								
Mean		83.7	92.1	91.3	92.6	87.6	88.9	85.2	95.5
RSD(%)		2.4	4.5	8.5	6.1	9.6	10.6	20.2	7.7
Canned soup	1								
Mean		81.5	87.5	80.5	95.0	89.8	80.4	75.4	96.1
RSD(%)		12.1	8.9	7.8	8.7	21.7	6.4	7.5	5.7
Canned soup	10								
Mean		87.3	91.4	89.3	91.9	89.7	90.6	88.8	94.0
RSD(%)		6.3	5.3	2.9	3.8	5.6	2.8	2.9	2.6

[*Isomeric mix; **3-methylacetophenone used to monitor distillation efficiency]

Table 3. cont.

Matrix	Spike level (mg/kg)	Menthofuran	Estragole	Pulegone	Safrole	*Cis*-isosafrole*	*Trans*-isosafrole*	Methyl eugenol	3-MA**
Soft drink	1								
Mean		77.5	77.6	71.3	75.0	72.4	71.8	80.9	91.5
RSD(%)		6.7	1.5	3.4	3.0	4.4	3.9	0.7	1.0
Soft drink	10								
Mean		81.4	85.8	84.2	93.2	90.5	91.6	101.9	87.2
RSD(%)		7.0	8.4	12.3	5.3	4.6	5.7	16.0	14.6
Veg. product	1								
Mean		77.9	104.7	105.5	97.2	95.7	93.0	86.0	99.5
RSD(%)		9.7	5.1	14.2	3.3	4.8	4.1	8.6	8.0
Veg. product	10								
Mean		71.6	74.8	90.7	76.7	72.4	68.5	67.9	77.4
RSD(%)		2.8	1.7	2.1	2.9	5.2	7.9	9.5	5.4
Chew. gum	25								
Mean		98.3	93.4	76.3	88.9	88.9	85.9	84.7	77.1
RSD(%)		3.6	2.0	13.3	3.1	3.1	3.4	4.7	12.1
Chew. gum	250								
Mean		102.9	90.8	84.2	86.0	84.2	85.5	84.0	93.8
RSD(%)		4.1	3.1	4.0	3.4	3.2	3.2	2.5	6.0
Fish prod.	1								
Mean		57.4	(1)	94.8	112.8	81.4	89.5	90.7	106.4
RSD(%)		9.6		3.2	3.7	1.7	6.1	6.8	11.1
Fish prod.	10								
Mean		82.9	143.9(2)	75.7	74.1	62.2	58.4	54.2	79.6
RSD(%)		11.3	22.0	36.5	40.8	52.6	65.7	81.3	33.7

[*Isomeric mix; **3-methylacetophenone used to monitor distillation efficiency. (1) High background level of estragole found ca 8.2 mg/kg recovery not calculated. (2) As for (1) except recovery not corrected for background estragole]

Table 4. Cross validation results for volatile BAPs by SDE

	BAP (mg/kg)					
Sample	Menthofuran	Estragole	Pulegone	Safrole	Isosafrole	Methyleugenol
Breath freshener	ND (22.1)	29.3 (13.6)	1.1 (20.8)	ND (ND)	ND (ND)	ND (ND)
Chewing gum	9.7 (33.5)	1.0 (0.9)	6.7 (29.3)	ND (ND)	ND (ND)	ND (ND)
Fish product	ND (1.0)	8.2 (4.8)	ND (1.0)	ND (ND)	ND (ND)	ND (0.1)
Ginger cordial*	ND (0.2)	123.2 (ND)	0.7 (0.2)	ND (0.2)	ND (ND)	0.8 (0.5)
Herbal tea	0.1 (ND)	120.7 (177)	0.8 (0.9)	ND (ND)	0.4 (0.3)	0.9 (1.0)
Mint confectionery	ND (0.9)	8.2 (0.3)	0.8 (8.2)	ND (ND)	ND (ND)	ND (ND)

[IL results in parentheses; ND = Not detected; Spiked with menthofuran, methyleugenol and safrole at 2, 1 and 1 mg/L respectively – all other samples unspiked.]

Table 5. Coumarin and quassine single laboratory validation data by direct analysis (n=4)

Sample matrix	Spike (mg/kg)	Coumarin recovery (%)		Quassine recovery (%)	
		2	**20**	**2**	**20**
Bagel	*Mean*	170	110	95	100
	RSD(%)	18.6	3.0	2.7	3.5
Breakfast cereal	*Mean*	108	99	99	93
	RSD(%)	12.6	1.3	6.5	15.0
Rice pudding	*Mean*	106	100	110	98
	RSD(%)	10.4	1.4	5.7	1.6
Gelatine confectionery	*Mean*	104	101	121	111
	RSD(%)	4.1	1.5	9.4	3.7
Biscuit	*Mean*	97	104	135	111
	RSD(%)	2.5	1.4	9.2	2.0
Sugar confectionery	*Mean*	102	102	108	106
	RSD(%)	2.3	1.5	8.4	2.0
Tonic water	*Mean*	99	99	95	99
	RSD(%)	7.1	1.4	16.2	1.2
Fruit flavoured drink	*Mean*	100	98	99	99
	RSD(%)	1.2	1.2	6.8	2.2
Camomile infusion		**Analyte peaks not discernible due to co-extractive interference**			
	Spike (mg/kg)	**200**	**2000**	**200**	**2000**
Mixed spice	**Mean**	116	107	113	109
	RSD(%)	4.0	2.3	7.2	3.3

Table 6. Coumarin and quassine single-laboratory validation data following clean up using SPE (n=4)

Sample matrix	Spike (mg/kg)	Coumarin recovery (%)		Quassine recovery (%)	
		2	**20**	**2**	**20**
Bagel	*Mean*	95	91	106	93
	RSD(%)	7.5	3.2	9.2	2.5
Breakfast cereal	*Mean*	92	90	104	110
	RSD(%)	6.0	2.7	10.3	19.6
Rice pudding	*Mean*	108	104	130	125
	RSD(%)	3.6	3.6	8.0	22.1
Gelatine confectionery	*Mean*	99	96	122	113
	RSD(%)	1.5	2.7	4.2	8.3
Biscuit	*Mean*	AF	AF	AF	AF
	RSD(%)				
Sugar confectionery	*Mean*	109	104	118	103
	RSD(%)	6.2	1.4	11.0	1.9
Tonic water	*Mean*	NA	NA	NA	NA
	RSD(%)				
Fruit flavoured drink	*Mean*	NA	NA	NA	NA
	RSD(%)				
Camomile infusion		**Analyte peaks not discernible due to co-extractive interference**			
	Spike (mg/kg)	**200**	**2000**	**200**	**2000**
Mixed spice	**Mean**	80	99	95	118
	RSD(%)	6.8	11.6	3.2	10.6

Table 7. Replicate analysis of coumarin-containing foods and quassine-spiked food (n=8)

Sample	**Mean coumarin content (mg/kg)**	**RSD(%)**
Cinnamon powder	2757	2
Cinnamon powder 'ground'	2495	1
Caramelised biscuit	10	6
Cinnamon bagel	27	2
Cinnamon balls (sugar confectionery)	19	2
Cinnamon Tatties (flour confectionery)	21	1
Cinnamon jelly beans	20	2
Cherry cinnamon tea (bags)	42	5
Orange and cinnamon tea (bags)	71	6
Rice pudding with added cinnamon	18	3
Cinnamon flavoured breakfast cereal	32	2
Cinnamon flavoured cookies	30	3
Quassine spikes	**Mean quassine content (mg/kg)**	**RSD(%)**
Cinnamon powder	3356	14
Caramelised biscuit	46	9

Table 8. Coumarin and quassine analysis cross-validation.
(IL results in parentheses)

Matrix	Added (mg/kg)		Found (mg/kg)		Recovery (%)	
	Coumarin	Quassine	Coumarin	Quassine	Coumarin	Quassine
Soft drink	11.2	0.6	11.6 *(10.7)*	0.6 *(ND)*	104	100
Rice pudding	5.0	0	5.5 *(5.4)*	0 *(1.5)*	110	NA
Cinn. bagel	0	1.1	22.0 *(24.5)*	1.1 *(ND)*	NA	100
Cinn. biscuit	0	1.0	0.6 *(0.6)*	3.0 *(ND)*	NA	300*
Break. cereal	9.4	0	9.7 *(11.3)*	0 *(ND)*	103	NA
Curry paste	0	0	40.5 *(33.2)*	0 *(ND)*	NA	NA
Herbal infusion	0	0	0 *(96)*	0 *(ND)*	NA	NA

[NA = Not applicable; ND = Not determined; * = Interfering peak(s) during HPLC]

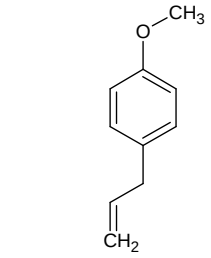
Table 9. HCN single laboratory validation data

Matrix	Sample wt/vol	Spike level mg/kg or /L HCN_{eq}	Mean recovery (%)	RSD (%)	Control matrix mg/kg or /L HCN_{eq}
Canned stone fruit	5g	1.05	98.9	23.9	2.3 (12.8)*
	5g	5.23	95.2	12.7	
Marzipan	1g	5.23	59.9	13.9	5.8 (3.8)*
	1g	52.3	65.5	13.7	
Alcoholic beverage	5mL	5.23	117.2	3.2	0.2 (7.3)*
	5mL	36.64	99.2	0.8	

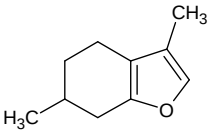
[* RSD(%)]

FIGURES

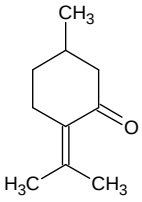
Figure 1. Chemical structures of BAPs and associated chemicals



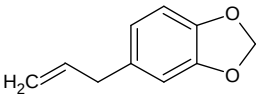
Estragole



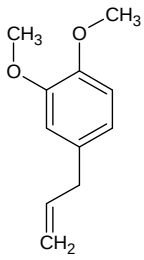
Menthofuran



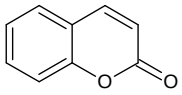
Pulegone



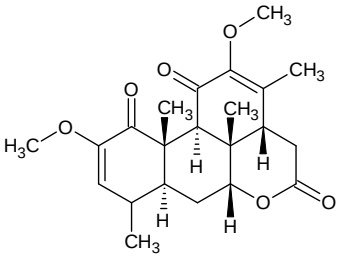
Safrole



Methyleugenol

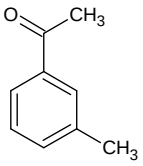


Coumarin

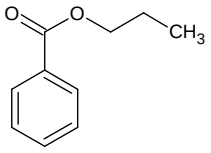


Quassine

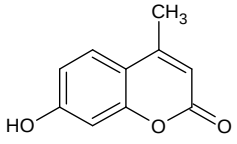
Hydrocyanic acid



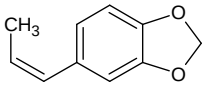
Methylacetophenone



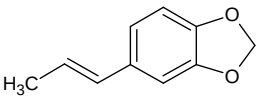
Propylbenzoate



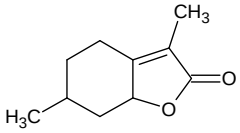
Methyl umbelliferone



cis-Isosafrole



trans-Isosafrole



Menthalactone

Figure 2. SDE apparatus

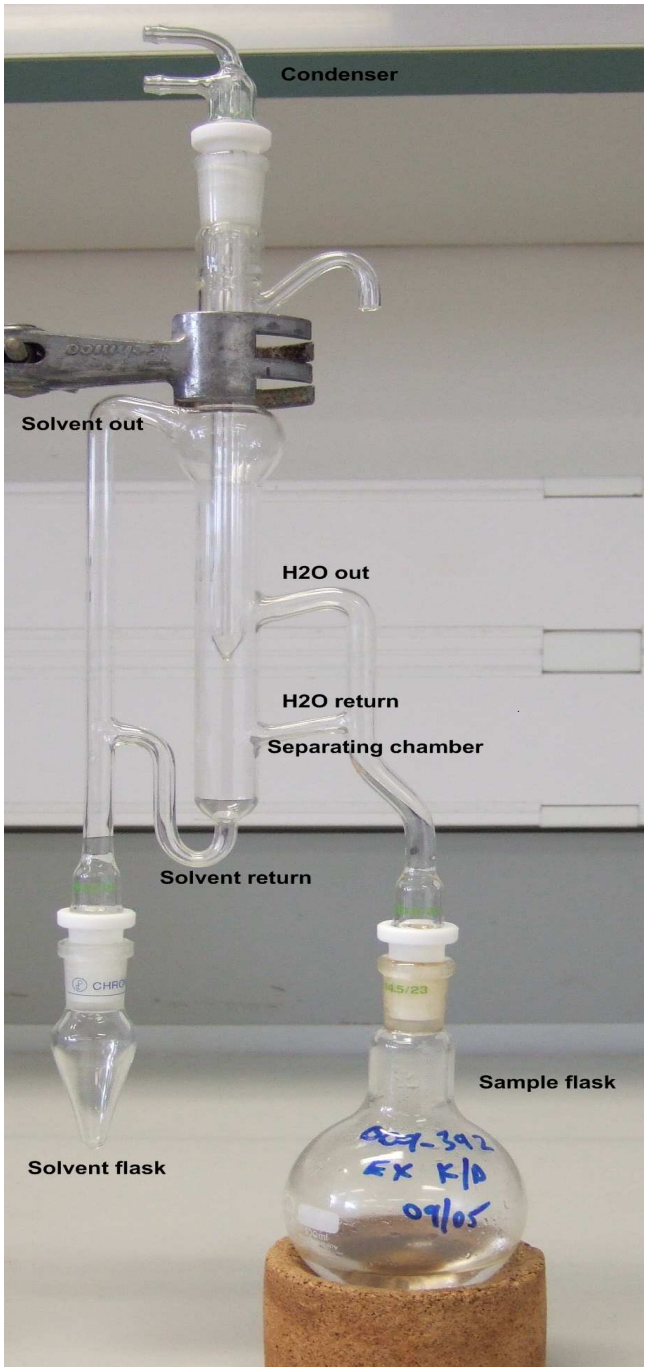


Figure 3. SDE-GCMS calibration graph

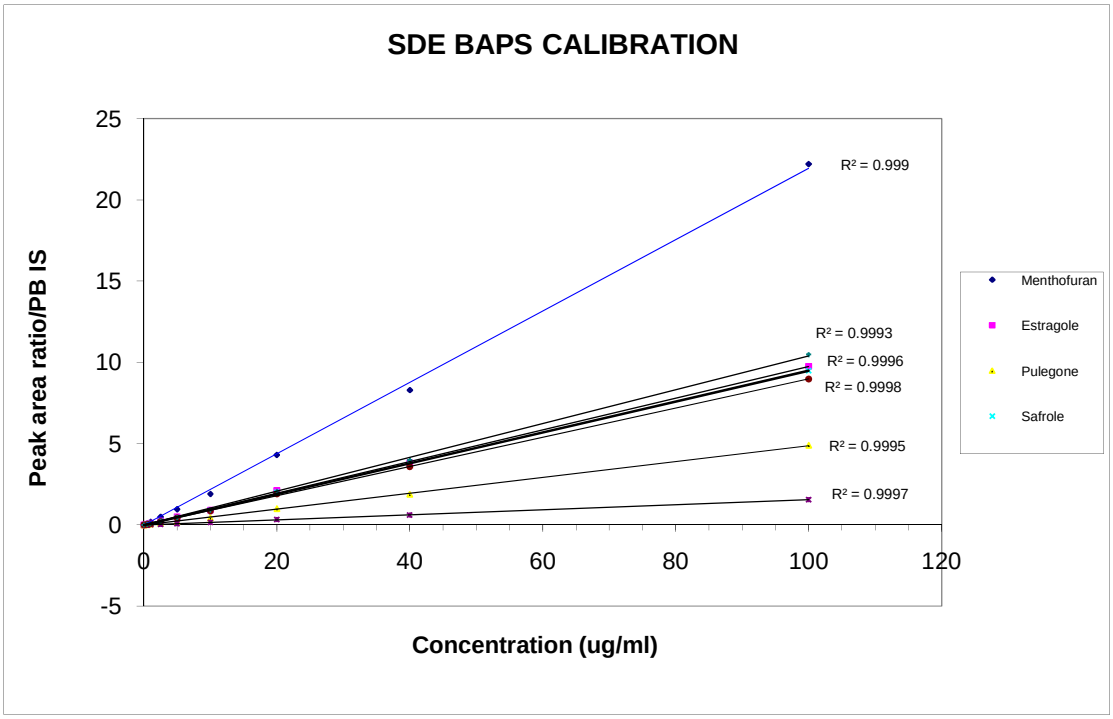


Figure 4. GC-MS TIC BAPs standard mix

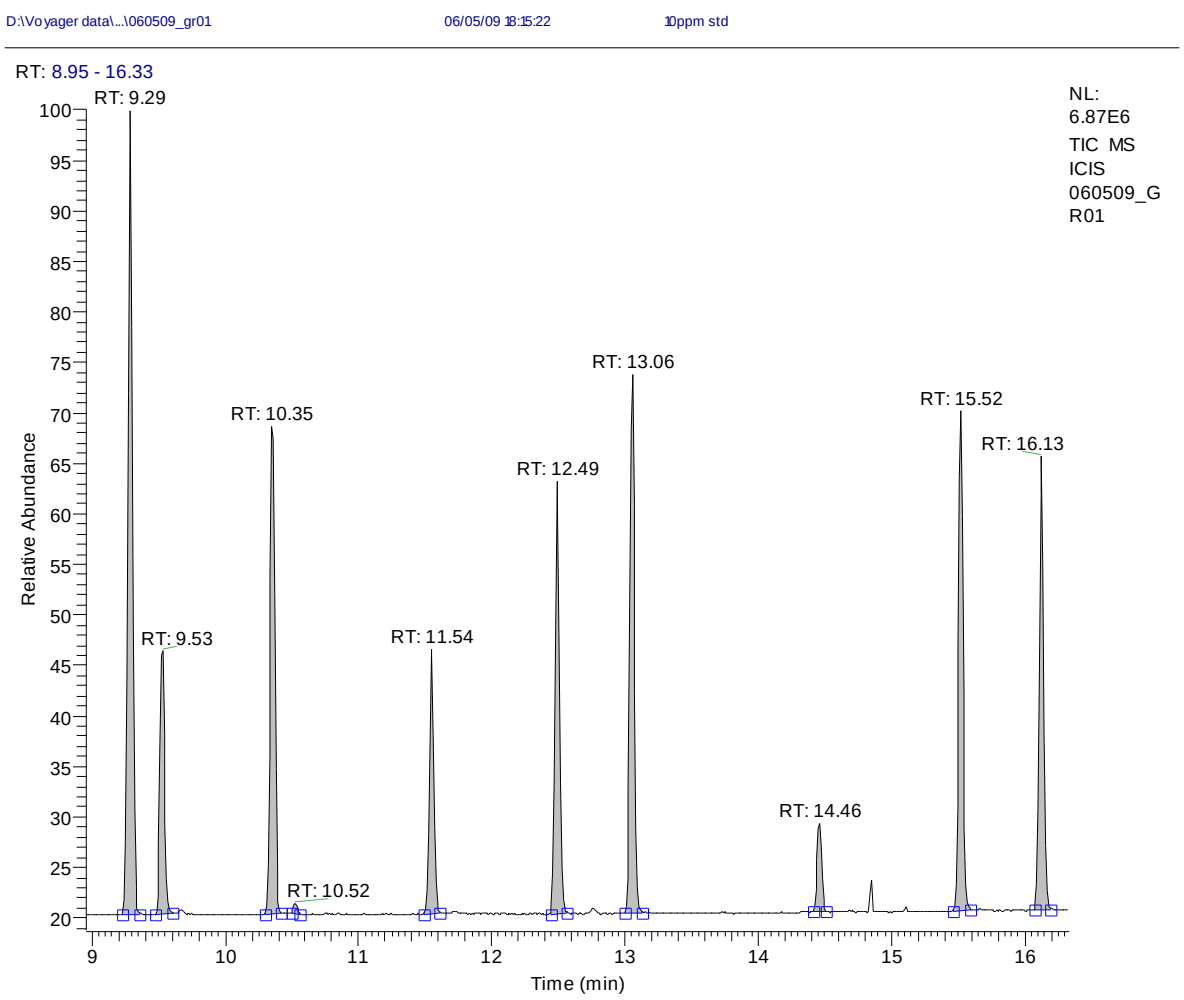


Figure 5. GC-MS SIM BAPs standard mix

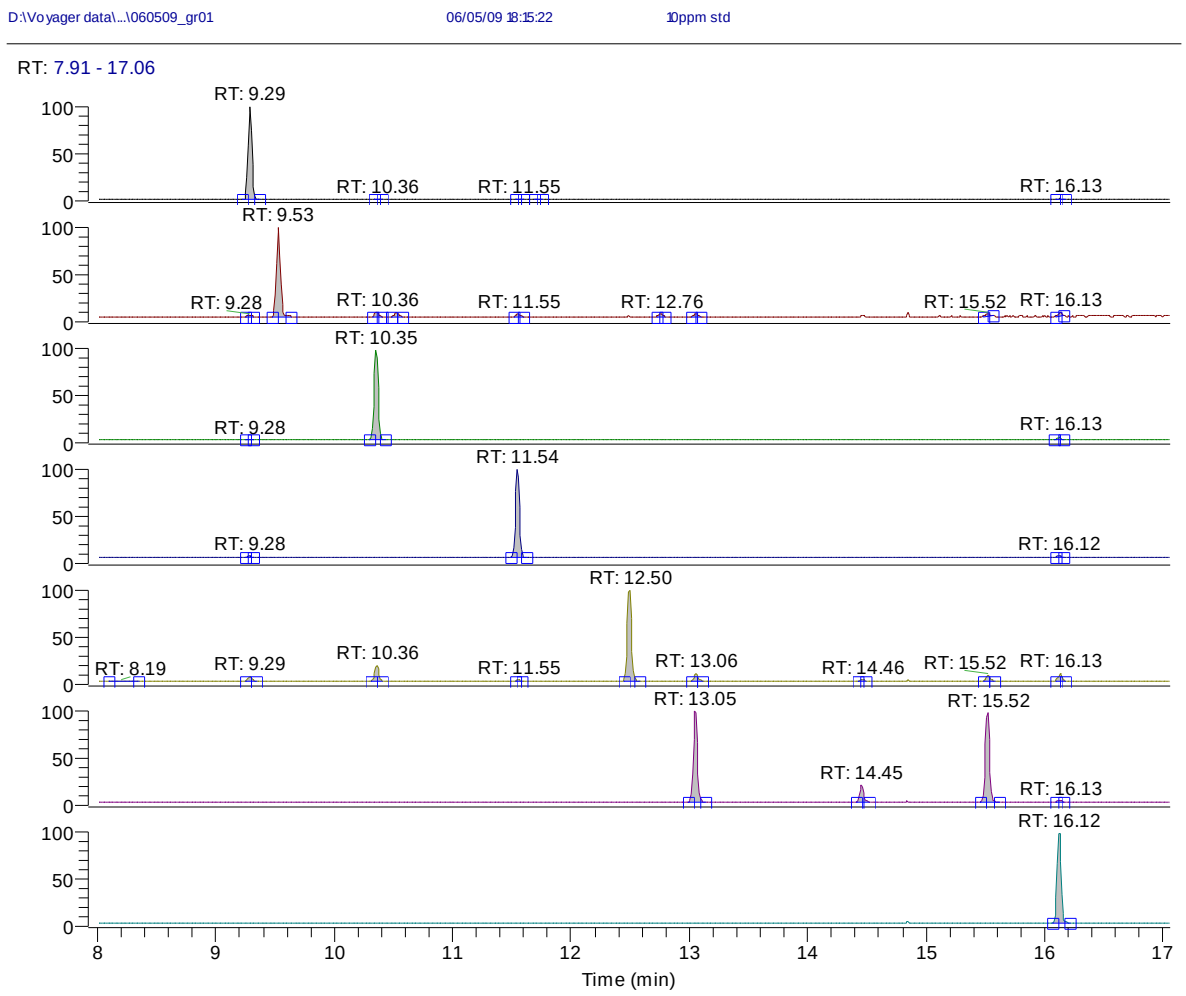


Figure 6. GC-MS (SIM) of unspiked mint flavour concentrate SDE extract. Note that this chromatogram was obtained before the GC temperature programme was finalised hence the retention times for BAPs are longer than those shown in Figure 5: Menthofuran 17.27 min, Estragole 18.33 min and Pulegone 19.48 min.

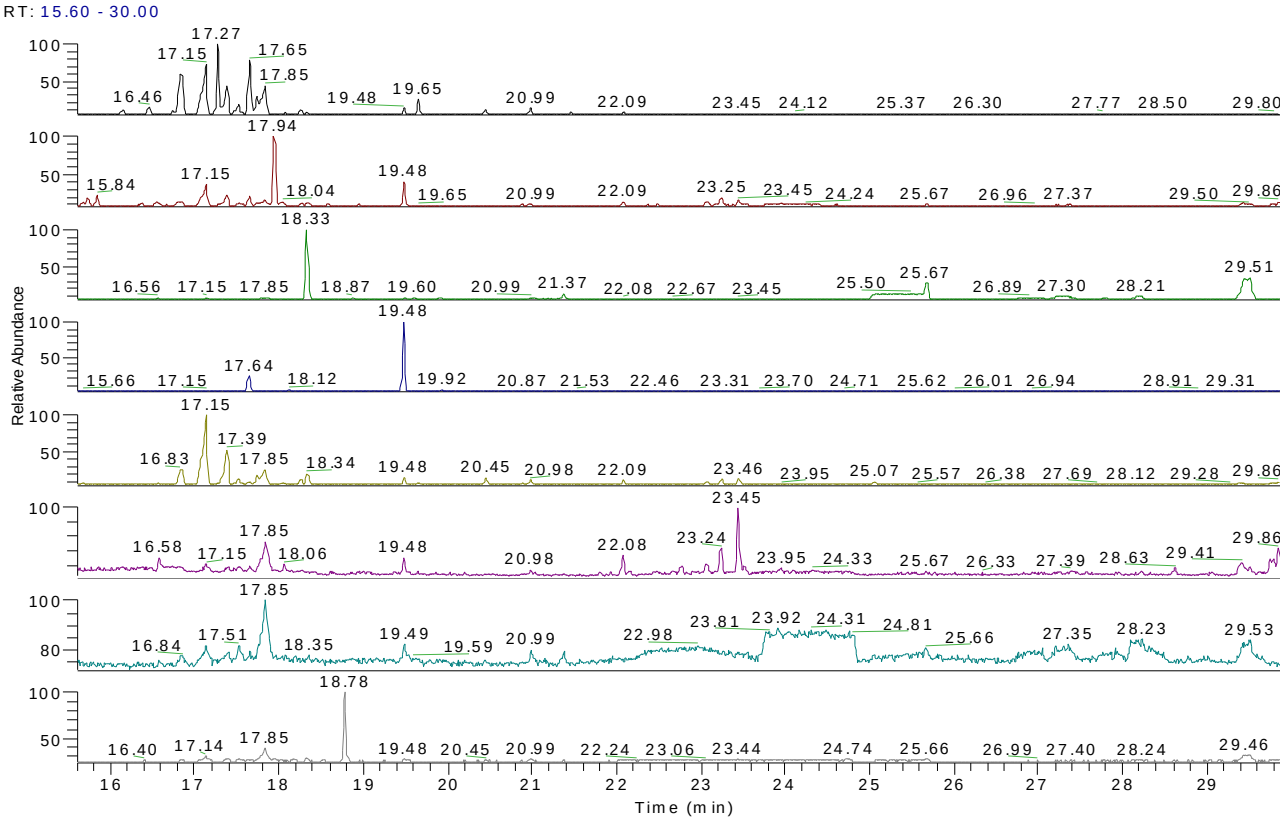


Figure 7. GC-MS (TIC) of SDE extract of flavoured crisps spiked (top), blank (middle) and standard mix (bottom). Note that this chromatogram was obtained before the GC temperature programme was finalised hence the retention times for BAPs are longer than those shown in Figure 5 and the spiking mixture did not contain menthofuran.

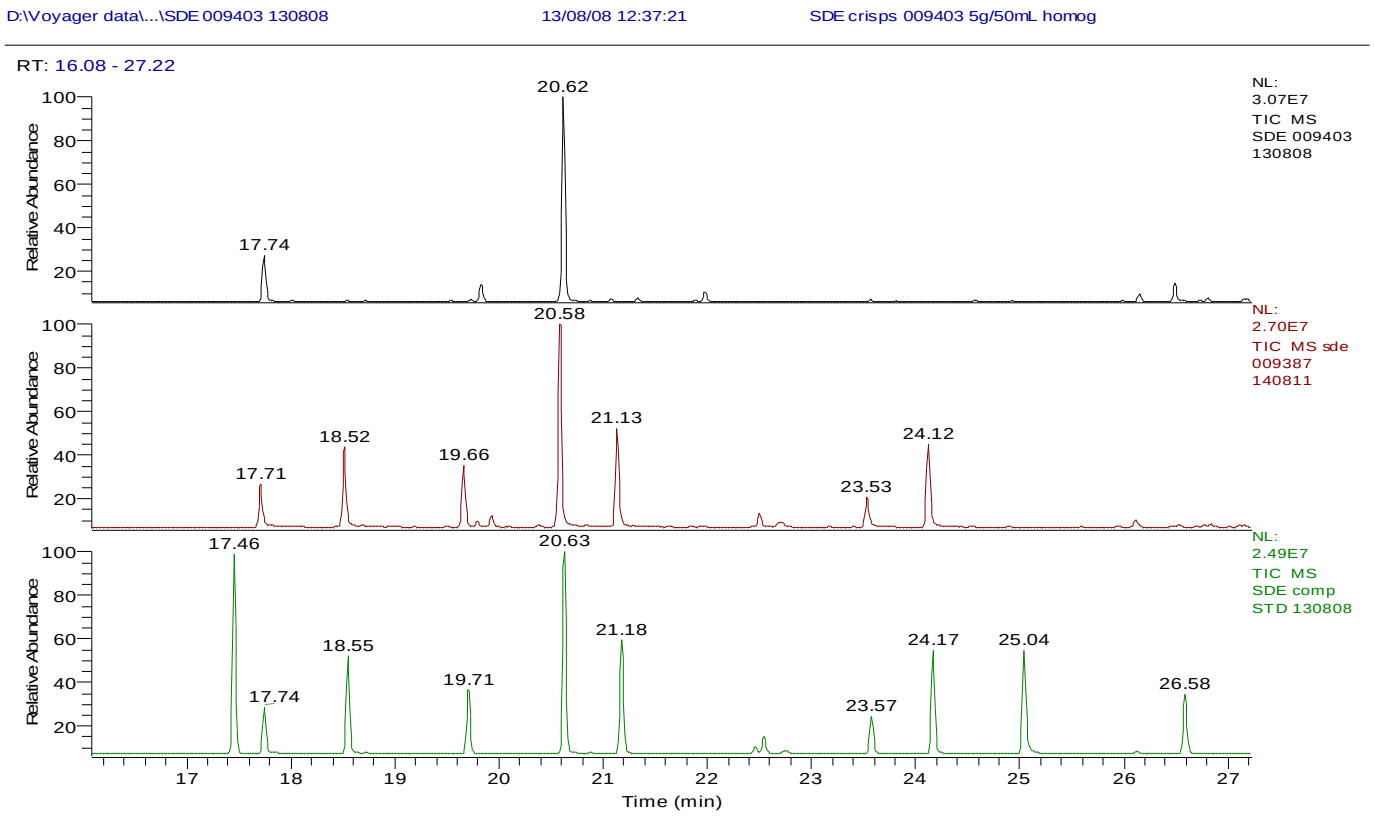
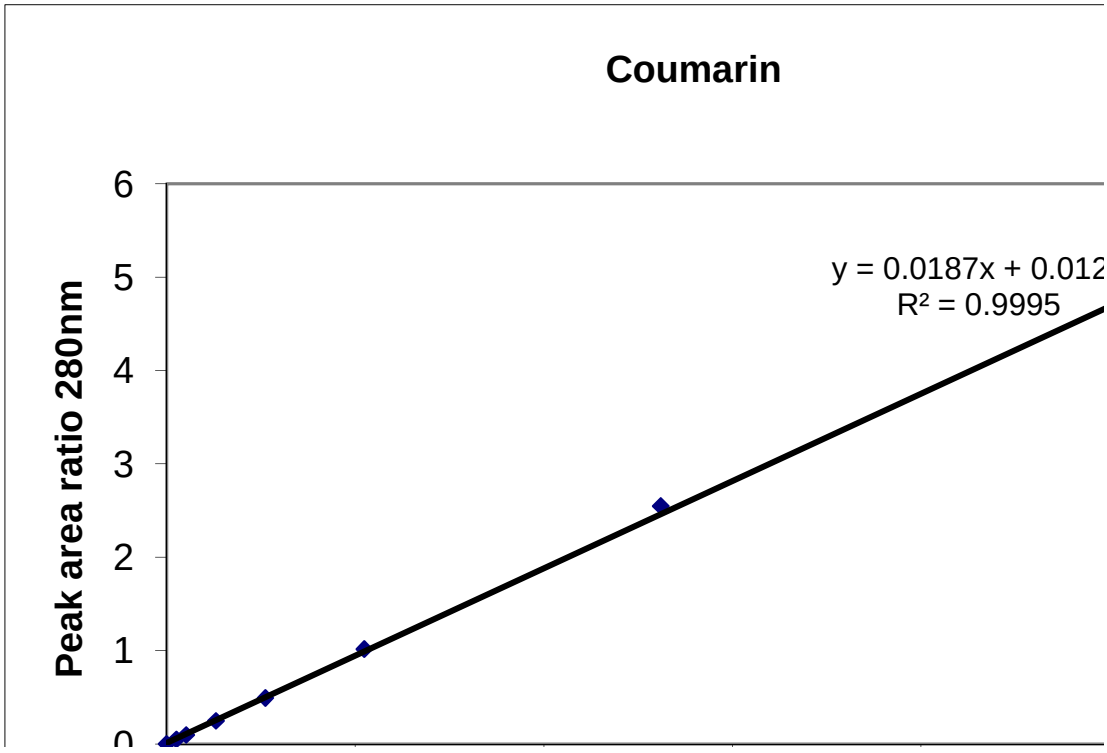


Figure 8. Coumarin and quassine calibration graphs for solvent standards



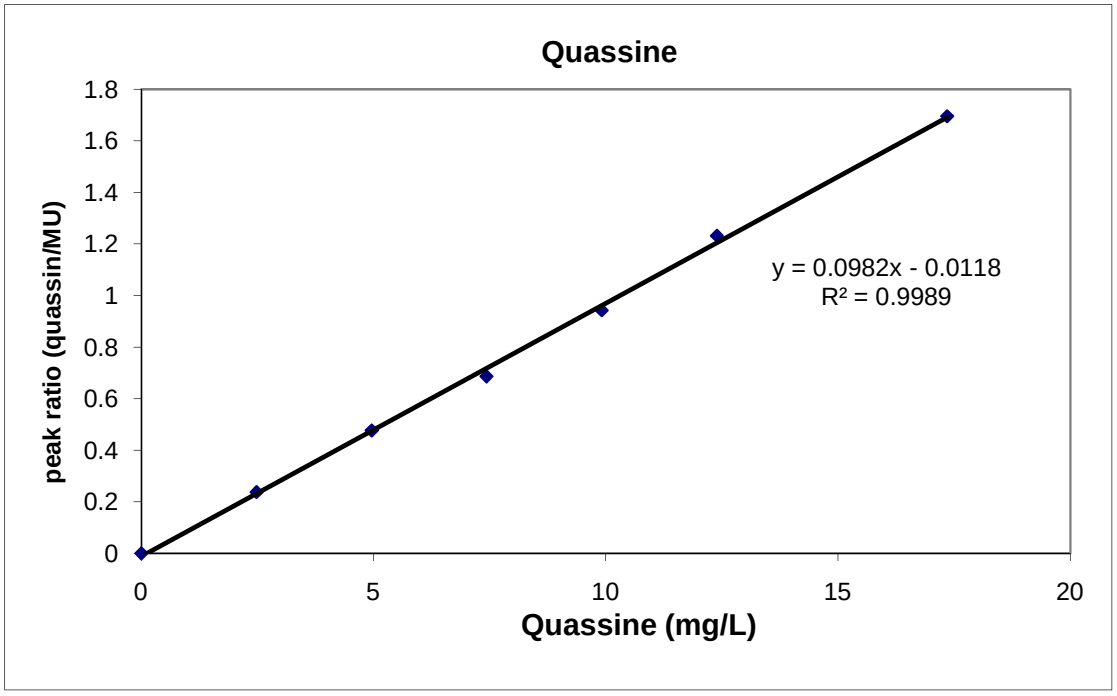


Figure 9. Coumarin calibration for spiked caramel biscuit

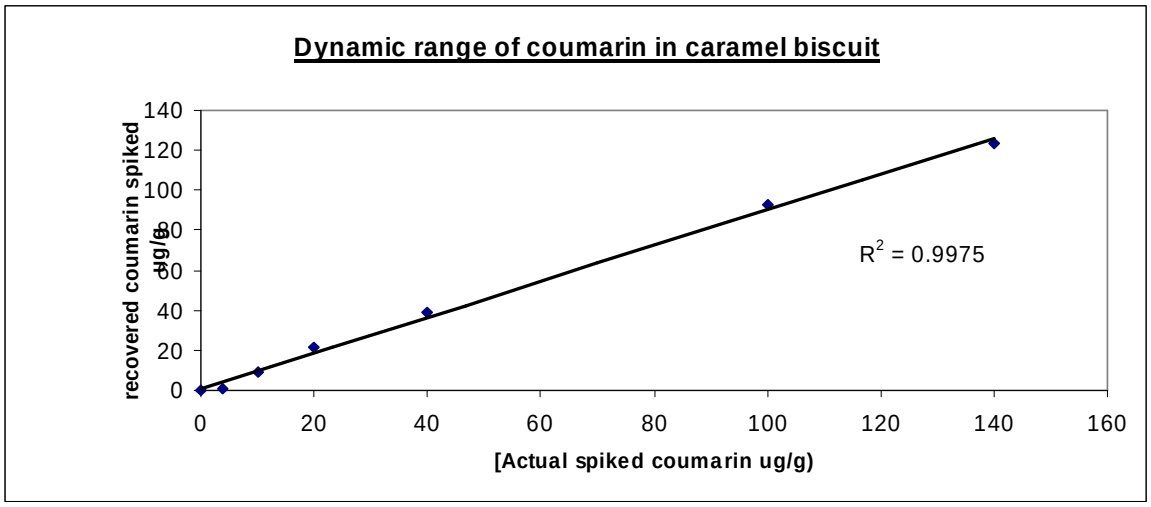


Figure 10. HPLC of cinnamon powder extract spiked with quassine

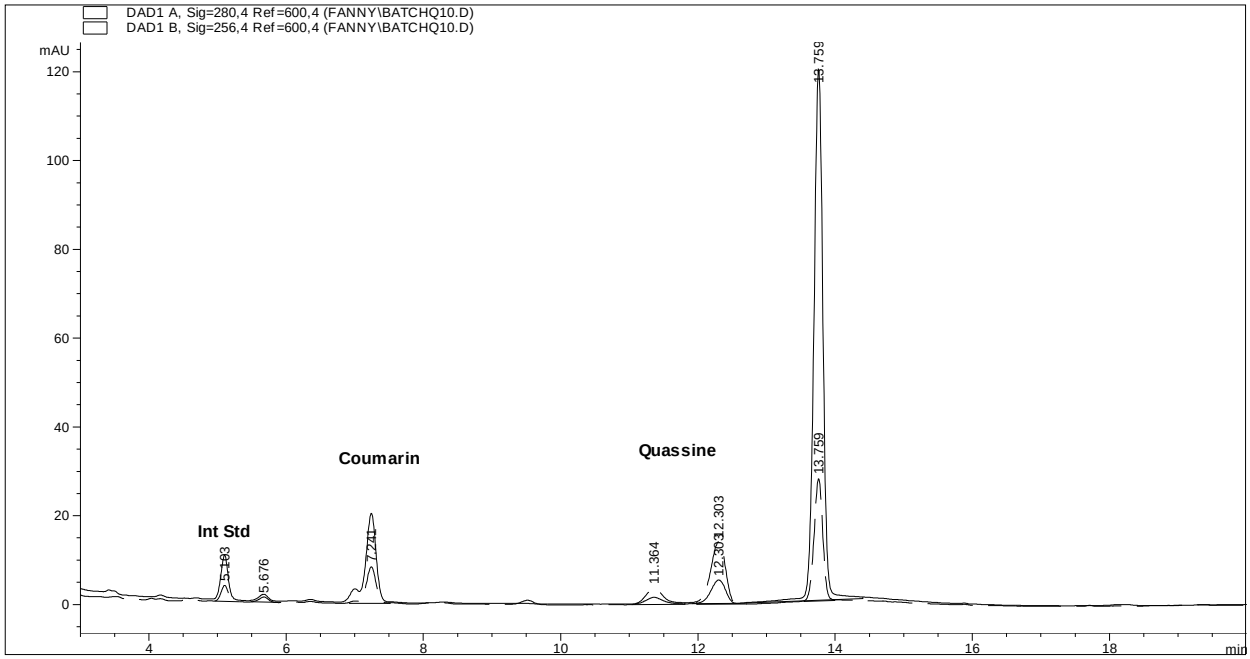


Figure 11. HPLC-PDA of camomile tea extract

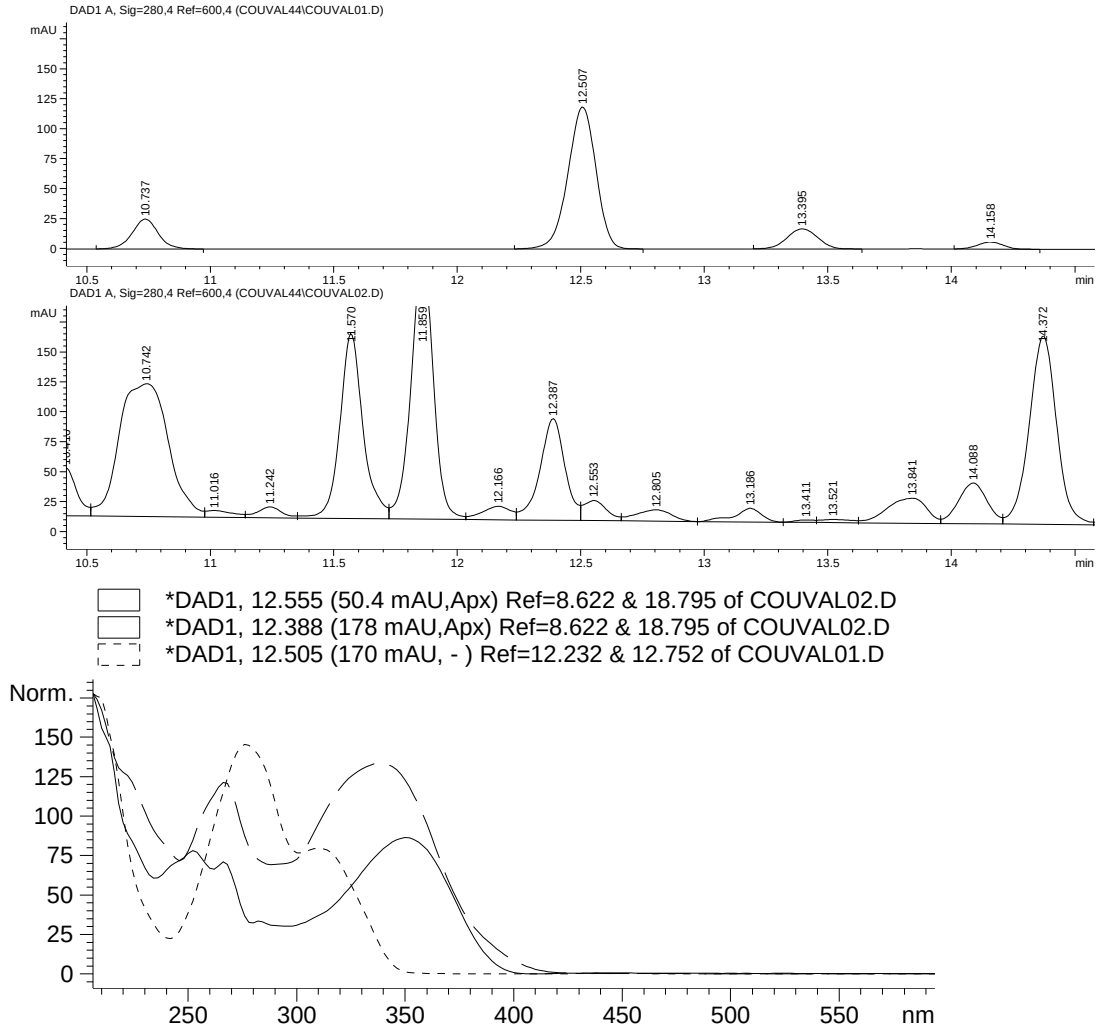


Figure 12. Hydrolysis of amygdalin to hydrogen cyanide

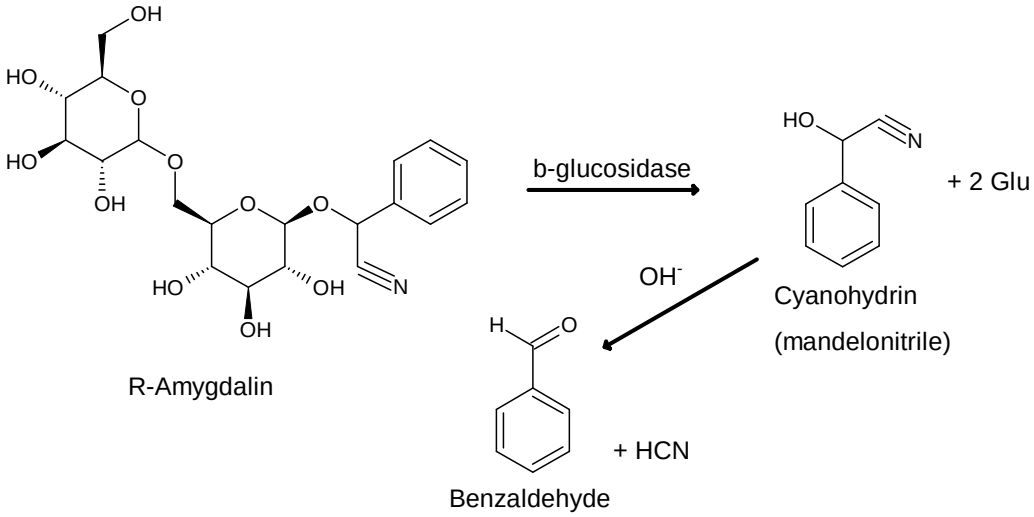
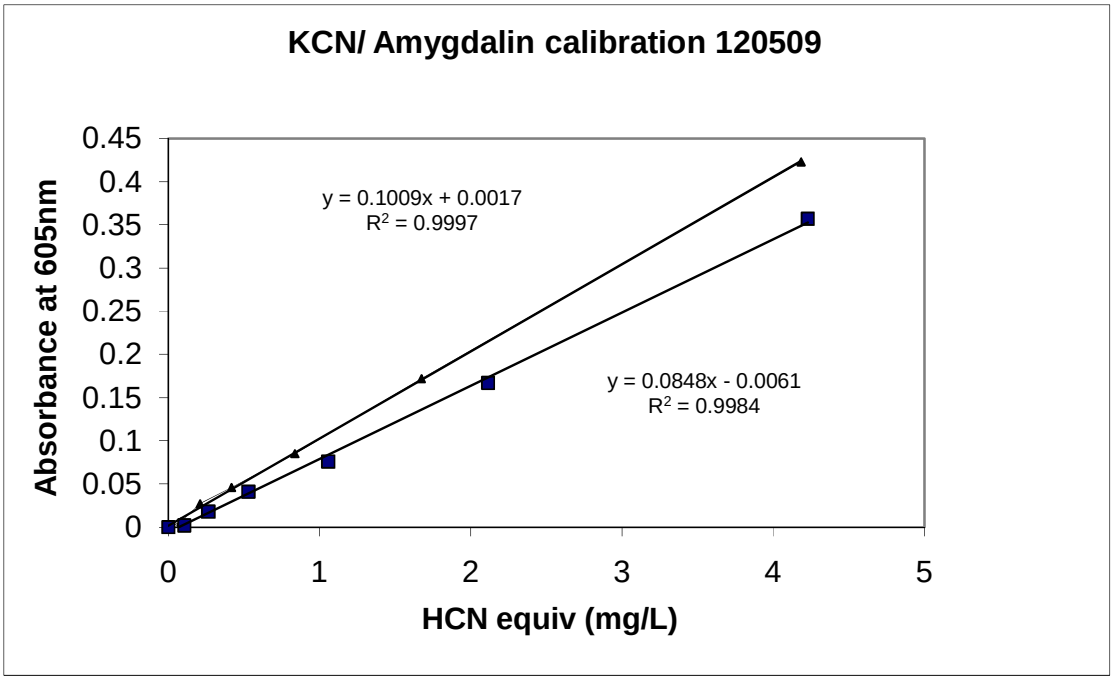


Figure 13. Comparison of KCN and amygdalin calibration



[KCN= ▲; Amygdalin = ■]

Figure 14. HCN colour development time plot