Potential natural sources of semicarbazide in honey

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Summary

Numerous databases were searched for information regarding the natural formation of semicarbazide (SEM), and for the presence of known and potential precursors of SEM in honey, particularly heather honey and in heather plants. Several publications describe the major components of heather and other honeys, such as amino acids and sugars along with other compounds such as phenols and flavonoids, but data on the minor components are limited to volatile compounds.

Studies have demonstrated the natural formation of SEM in certain shellfish, seaweed, eggs, and whey. Certain nitrogenous compounds, principally the amino acid arginine, were proposed as precursors of SEM in these foods. Arginine has been reported to be present at a high level in shellfish for example. The literature reports that arginine is present in heather honey but not at levels higher than in other types of honey. However, a single interesting paper on the analysis of dew collected in traps showed that levels of some amino acids, notably arginine, reached very high proportions on certain occasions. This suggests that arginine levels could have been elevated shortly before and during the production of the affected honey.

Other possible sources of the SEM reported in heather honey are as-yet unidentified precursors, environmental contaminants or the illicit use of nitrofurazones or other nitrofuran antibiotics which were not detected by the analytical methods used. However, the absence of other metabolites of nitrofurans and the known provenance of the honey contradict this.

Further investigation would benefit from supporting analyses monitoring other metabolites of nitrofurazone/nitrofurans using alternative methods, a more thorough investigation of possible precursors in the heather plants (particularly arginine), and laboratory attempts to enhance the production of SEM in heather and heather honey from some amino acids.

Field studies to monitor SEM and arginine in heather honey would give a clearer picture of the situation but would be expensive as long-term sampling and many samples would be required. Laboratory studies could provide an alternative method for SEM analysis that would distinguish between natural formation and production from nitrofurazones based on the fact that nitrofurazones produce markers other than SEM, and several experiments could provide information on the precursors of SEM and its formation mechanisms, possibly allowing mitigation activities. Studies of SEM and precursors in plants, bees and honey maintained at an experimental facility would give confidence in the results on account of the statistically significant sample numbers and greatly reduce the costs of sample collection. These approaches would give the best value.

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Introduction

The aim of this review was to investigate whether there is any evidence for potential sources of semicarbazide (SEM) in honey and whether it could be produced by natural processes, especially in high purity heather honey.

SEM has been found to occur in several different types of food and currently there are five known chemical sources of this compound in foods. SEM can be present in foods as:

1. A metabolite of the veterinary antibiotic nitrofurazone which is genotoxic to humans and as such in Europe, is a prohibited substance for all food-producing animals under Commission Regulation No 37/2010 (EU 2010). SEM is consequently used as an indicator in tests to detect the use of nitrofurazone in food-producing animals In the European Union; SEM in food should not exceed the Minimum Required Performance Limit (MRPL) of 1µg/kg as defined in Annex II of Commission Decision 2002/657/EC.
2. A migration product from a thermal breakdown of azodicarbonamide (ADC), a blowing agent used to foam the plastic sealing gaskets in metal lids on jars or bottles. The use of ADC for food contact materials was banned by the European Commission in 2005.
3. A decomposition product of ADC used as a flour treatment agent (dough–improver) in bread production. This use of ADC to treat flour is not permitted in the EU.
4. A reaction product formed between hypochlorite (‘bleach’) and food additives such as carrageenan and foods such as powdered egg white.
5. A compound present at background levels in some foods, such as crayfish, shrimps and prawns. The Veterinary Residues Committee has recently concluded that SEM can occur naturally in soft shell crab (Veterinary Residues Committee 2012).

In 2010, the detection of SEM in samples of good quality heather honey of high purity, mainly from Scottish hives which were unlikely to have been treated with nitrofurazone, suggests that SEM may have originated from another (unidentified) source. The European Food Safety Agency (EFSA) has concluded that SEM is not a concern for human health at levels found in food (EFSA 2005), but the presence of SEM in honey could adversely affect consumer confidence in this product and severely damage the honey industry.

The uncertainty about SEM as a reliable marker of nitrofurazone use in honey production needs to be addressed in order to protect consumer safety and confidence in the product.

Results of honey analyses were provided to the Food Standards Agency in Scotland (FSAS) by bee farmers. A consultant analytical laboratory with UKAS accreditation analysed 13 honey samples in 2010 for the nitrofuran metabolites 3-amino-2-oxazolidone (AOZ),

3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), 1-aminohydantoin (AHD), and SEM by a high performance liquid chromatography/tandem mass spectrometric method (LC-MS/MS). Two samples were also subjected to pollen analysis. SEM, but no other metabolites were detected in several samples.

The 2010 crop of Scottish heather honey had been found to contain SEM, with confidence that it had not arisen from use of nitrofurans. Dozens of samples from different beekeepers and many different apiary locations had been tested, with all heather honey samples showing the presence of SEM, at consistent levels between 0.6 and 1.8 µg/kg. Samples of other honeys, produced from the same hives but at different times and/or locations, did not show SEM, suggesting a link between SEM and heather honey.

In 2010 SEM has also been reported in a sample of honey collected in England and SEM has recently been reported in two samples of wild forest honey (Kamahi) honey imported to the UK from New Zealand, one of which has been reported formally (VMD 2012). The New Zealand Ministry of Agriculture and Forestry do not believe that nitrofurazone was used in the beehive in question and that they are investigating this possible natural occurrence.

It was assumed that the consistent pattern of SEM occurrence in the Scottish heather honey suggests an underlying reason for the presence of SEM, possibly due to botanical peculiarities with heather nectar. It was reasoned that nitrofuran use would have given far more variable results with higher levels and some samples with SEM not detected.

Reasons for the positive finding of SEM in heather honey.

There are several potential reasons for the positive finding of SEM in heather honey:

1. SEM was present in the heather honey through transfer from *Calluna* plants.
2. Natural SEM precursors were present in the heather honey through transfer from *Calluna* plants, possibly contaminated by sheep's urine.
3. Natural or non-natural SEM precursors were collected by the bees from other sources.
4. The bees' metabolic processes can produce SEM from precursors present in heather.
5. Conditions in the hive can lead to formation of SEM from precursors in the honey.
6. Natural or non-natural SEM precursors were present in the heather honey through environmental contamination.
7. The honey samples were contaminated through packaging.
8. The honey samples were contaminated with SEM derived from bleaching activities.
9. The test results were falsely positive.

10. Nitrofurans were used on the hives producing the honey.
11. A different non-natural class of SEM precursor was applied to the hives.
12. SEM was present in a recycled beeswax-foundation added to the hive.

This review focuses on the potential for the formation of SEM in heather and other honeys in the absence of nitrofurazone/nitrofurans contamination.

Literature searches

Initial searches were carried out using 249 'science' databases, which in fact included many general media and patents databases etc. In-depth searching was in most cases carried out on the 11 major and most relevant databases listed below.

Biosis Previews(R) 1926-2012 The Thomson Corporation.
SciSearch(R) Cited Ref Sci 1990-2012 The Thomson Corp.
Food Sci.&Tech.Abs 1969-2012 FSTA IFIS Publishing.
FOODLINE(R) Science 1972-2012 LFRA.
EMBASE 1974-2012 Elsevier B.V.
PIRA 1975-2012 Pira International.
CA SEARCH(R) 1967-2012 American Chemical Society.
Derwent WPI 1963-2012 Thomson Reuters.
Business & Industry(R) Jul/1994-2012 Gale/Cengage.
Dialog Global Reporter 1997-2012 Dialog.
Chem Bus NewsBase 1984-2012 Elsevier.

Searches were aimed at finding information on:

1. Occurrence of SEM or precursors in heather plants.
2. Occurrence of SEM in heather honey and other honeys.
3. Natural SEM formation in other foods.
4. The chemical composition of heather, *Calluna vulgaris,* and *Erica* species.
5. Occurrence of amino acids, arginine and citrulline, in heather honey and other honeys.
6. Occurrence of other chemicals in heather honey and other honeys.
7. Occurrence of other nitrogenous compounds including creatine, creatinine, and urea in heather honey and other honeys.
8. Occurrence of arginine and citrulline in pollen and nectar.

9. Occurrence of arginine in crustaceans including crayfish, shrimp, prawns, crab, shellfish or seafood.

Review

Occurrence of SEM in honey

The Annual Reports on Surveillance for Veterinary Residues in Food in the UK published between 2005 and 2010 show no significant occurrence of positive results for SEM in UK honey apart from the results published in 2005 in which eight positive samples were reported from 100 tested. These were attributed to contamination of the honey by the plastic seals on the metal screw-tops used on jars, which as described above was an application banned in the EU in 2005.

Approximately 20 honeys from each of Belgium, Italy, Portugal, Spain, Switzerland, and the UK were tested in 2009 for the presence of nitrofuran metabolites (SEM, AMOZ, AOZ, and AHD), and all were found to be negative (Reybroeck and Ooghe 2010).

Formation of SEM

SEM is a derivative of hydrazine or urea, with the chemical structure shown in Figure 1.

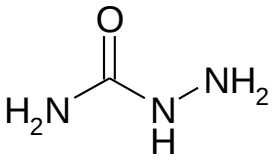


Figure 1 Structure of SEM

SEM can be formed by the Hofmann reaction between urea and hypochlorite at strongly alkaline pH (Bendall 2009). In industry and in the laboratory it is usually made from hydrazine and isocyanate, themselves made from hypochlorite and ammonia or amides. Hypochlorite also enhances greatly SEM formation in a number of foods and compounds found in foods.

Natural formation of SEM in foods.

Sources of SEM in food were reviewed by de la Calle and Anklam (2005) who summarised the sources as nitrofurazones, azodicarbonamide (ADC), and hypochlorite treated nitrogen-rich food ingredients. ADC is used in many countries outside the European Union but it is unlikely to have contaminated honey.

SEM has also been reported as present in the food additive carrageenan, a complex mixture of polysaccharides made from extracts of a red seaweed species of the Rhodophyceae (*Chondrus, Gracilaria, Solieria, Palmaria*, and *Euchema*). Carrageenan is used as a thickening agent (additive E407) in processed poultry and meats, dressings, ice cream, yoghurts, puddings, jellies and preserves. Several processing stages are used to isolate and purify carrageenan, including alkaline extraction, drying, and bleaching, and it has been shown that SEM is formed during these processing operations.

Hoenicke *et al.* (2004) reported SEM levels of between 1 and 3 µg/kg in some sun-dried red seaweed (Rhodophyceae) species. They considered that it could reasonably be assumed that SEM can be formed by the degradation of nitrogen-containing substances having either an amidino or ureido residue such as arginine, histidine, citrulline, creatine or creatinine. Arginine and citrulline are major components of the metabolic pathway known as the urea cycle. Structures of the proposed SEM precursors and SEM itself for comparison are shown in Figure 2.

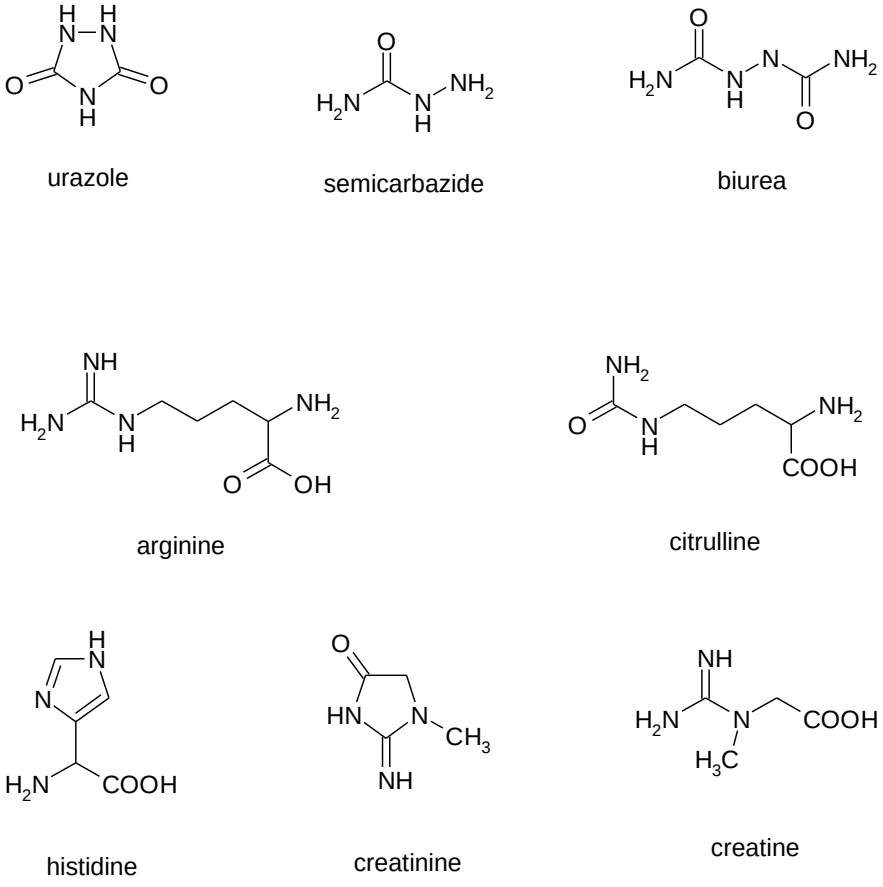


Figure 2 Structures of the proposed SEM precursors and their analogy to SEM

Hoenicke *et al.* (2004) also carried out a range of experiments to investigate SEM formation from selected foods and precursors. A range of foods and proposed precursors was incubated (but not heated) overnight with and without hypochlorite. No detectable SEM (< 0.2 µg/kg) was formed in the food categories fish, chicken, deer, and Parmesan cheese. However detectable levels up to 2.2 µg/kg were formed in red seaweed, shrimps, prawns, egg, milk powder, soya bean flakes and gelatine.

When naturally occurring potential SEM precursors were incubated in the same manner no SEM (< 0.2 µg/kg) was produced from histidine, creatine, citrulline, or urea, but arginine formed 1.5 µg/kg and creatinine formed 2.8 µg/kg SEM.

Gatermann *et al.* (2004) reported that SEM was formed in dried egg product and in whey simply during storage under warm conditions. Dried albumen contained about 100 µg/kg SEM on storage for one week at 70-80°C, increasing to about 300 µg/kg after five weeks. SEM also increased over five weeks in dried egg powder to a maximum of 10 µg/kg and in whey powder to 20 µg/kg under the same heating conditions. The levels of SEM formed were lower when the products were stored in the absence of oxygen.

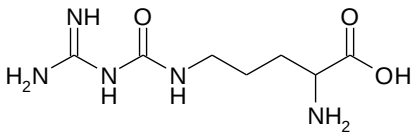
Arginine makes up 5-6% of the amino acid composition of hens' eggs (Lunven *et al.* 1973) and 2-3% of whey protein (Hambraeus 1982).

Natural presence of SEM in crustaceans.

Natural SEM precursors have been identified in the shell of marine crustaceans such as crayfish, shrimps, prawns and soft shell crab. These are relevant in that they might indicate a chemical formation route of SEM or help identify SEM precursors in honey or nectar sources. The first reports of possible natural occurrence in crustaceans were made by Saari and Peltonen (2004) and these were followed by studies carried out by van Poucke *et al.* (2011a, 2011b), and by McKracken *et al.* (2011). Saari and Peltonen detected SEM in all of 18 Finnish crayfish samples that had never been exposed to nitrofurazone, but interestingly did not find SEM in shrimp. The origin of the SEM was not identified. SEM in both free and protein-bound forms was measured by LC-MS/MS. The average total level of SEM was 4.2 µg/kg. The published evidence of identity of the SEM is convincing and no SEM was detected in blank control materials.

van Poucke *et al.* confirmed these findings using prawns raised in the laboratory and also analysed by LC-MS/MS. McKracken *et al.* (2011) reported that drying shrimp shells at 60°C overnight did not increase SEM and neither did exposure to bleach (hypochlorite). They also reported that SEM could be produced from the amino acid gigartinine (Figure 3) by conditions used in the analytical determination of SEM, i.e drying overnight at 60 °C, extraction with methanol/water, hydrolysis with dilute hydrochloric acid and derivatisation

with 2-nitro-benzaldehyde. Gigartinine is produced by species of red alga and has not been reported in heather or honey.



gigartinine

Figure 3 Structure of gigartinine

The European Union's Rapid Alert System for Food and Feed (RASFF) monitored SEM notifications between 2002 and 2009 and reported SEM in 183 samples of crustacean foods with SEM levels exceeding 1000 µg/kg in seven samples (Hruska and Franek 2009). They also reported a wide range of levels (0.4 to 19.3 µg/kg) in seven wild caught crustaceans.

Research at Ghent University (Belgium) and the Agri-Food & Biosciences Institute (Belfast, Northern Ireland) confirmed that SEM was present as a natural component primarily in the shell of a range of crustaceans including prawns, crabs and langoustines. Levels in shell were around 25 µg/kg compared to less than 0.5 µg/kg in the meat (SIPA 2010).

SEM was reported present in crayfish after cooking (Saari and Peltonen 2004) but uncooked samples were not analysed. The levels of SEM were reduced by washing, showing that it was not tissue-bound. However in prawns cultivated in nitrofurazone free conditions SEM is found almost entirely in bound forms in the shell tissue (van Poucke *et al.* 2011). No cooking conditions were described, but the prawn tissues were treated with 0.2M hydrochloric acid at 37°C overnight, which might have caused some SEM formation from precursors.

It has been established that the SEM in crayfish is restricted almost entirely to the shell. The location of arginine (proposed as a SEM precursor) in crayfish is less certain. However its presence at proportionately high levels has been found in some antimicrobial peptides produced by marine crustaceans and marine worms. They include penaeidins, crustins, arasin, aracin, aranecin, and hyastatin. These peptides are high in specific amino acids, mostly proline, arginine and cysteine. Penaeidins have a proline-arginine rich N-terminus and a cysteine-rich C-terminus. Aracins have a C-terminus containing cysteine and an N-terminus rich in proline and arginine, with positively charged arginine side chains. They bind to chitin and are active against Gram-positive or Gram-negative bacteria, sometimes both, as well as fungi (Smith *et al.* 2010).

Arginine in shellfish

Publications describing the amino acid composition of crustaceans show that levels of arginine are high in some species. Huong *et al.* (2001) showed that the glycine, arginine, alanine, proline and lysine content of freshwater prawn increased dramatically with salinity of the water. Ngoan *et al.* (2000) found that arginine was the most abundant amino acid in some shrimp species, and in prawns, crab, squid, and scallop. Several other publications support reports of the high level of arginine in marine crustaceans, which might be associated with natural SEM formation in the shell or cuticle.

Arginine, aspartic and glutamic acids accounted for 33% of the total amino acids of common shrimp species from Vietnam (Ngoan *et al.* 2000). Shrimps, prawns, crab, squid, and scallops were rich in arginine. The levels of arginine and glycine, but not other amino acids, changed with the season. (Lee *et al.* 1989)

Insect cuticle has only partially been characterised, however genome research has shown that two proteins contain an arginine-rich hydrophilic N-terminal region with a tryptophan C-terminal residue (Kucharski *et al.* 2007). It is possible that there is a high proportion of arginine in bee cuticle, which might possibly enter the honey or be converted to SEM.

Potential formation route for SEM

ADC breaks down to form biurea (hydrazodicarbonamide), SEM and urazole (Becalski *et al.* FAC 2004; Stadler *et al.* Analyst 2004). Pereira *et al.* (2004) and Noonan *et al.* (2008) proposed that the formation of SEM from ADC in bread involved biurea as an intermediate. Ye *et al.* (2011) confirmed that high temperature hydrolysis of biurea leads to SEM formation. When SEM is produced in bread made with ADC the yield of SEM is greatly increased when biurea is added to the wet flour. The hydrolysis of biurea requires considerable energy, which is unlikely to be available during commercial honey production processes.

Because Hoenicke *et al.* (2004) have demonstrated the formation of SEM from arginine and creatinine, and because SEM is known to be formed from biurea, then there is a possibility that arginine and creatinine form biurea as an intermediate step towards SEM according to the route proposed in Figure 4. Arginine can generate urea as in the urea cycle that takes place in insects. Oxidation of urea and reaction with a second urea molecule could produce azodicarbonamide, leading to SEM formation from known pathways.

The information obtained from searches for the natural occurrence of arginine in *Calluna*, in honey products and in shellfish gives some support to this proposal.

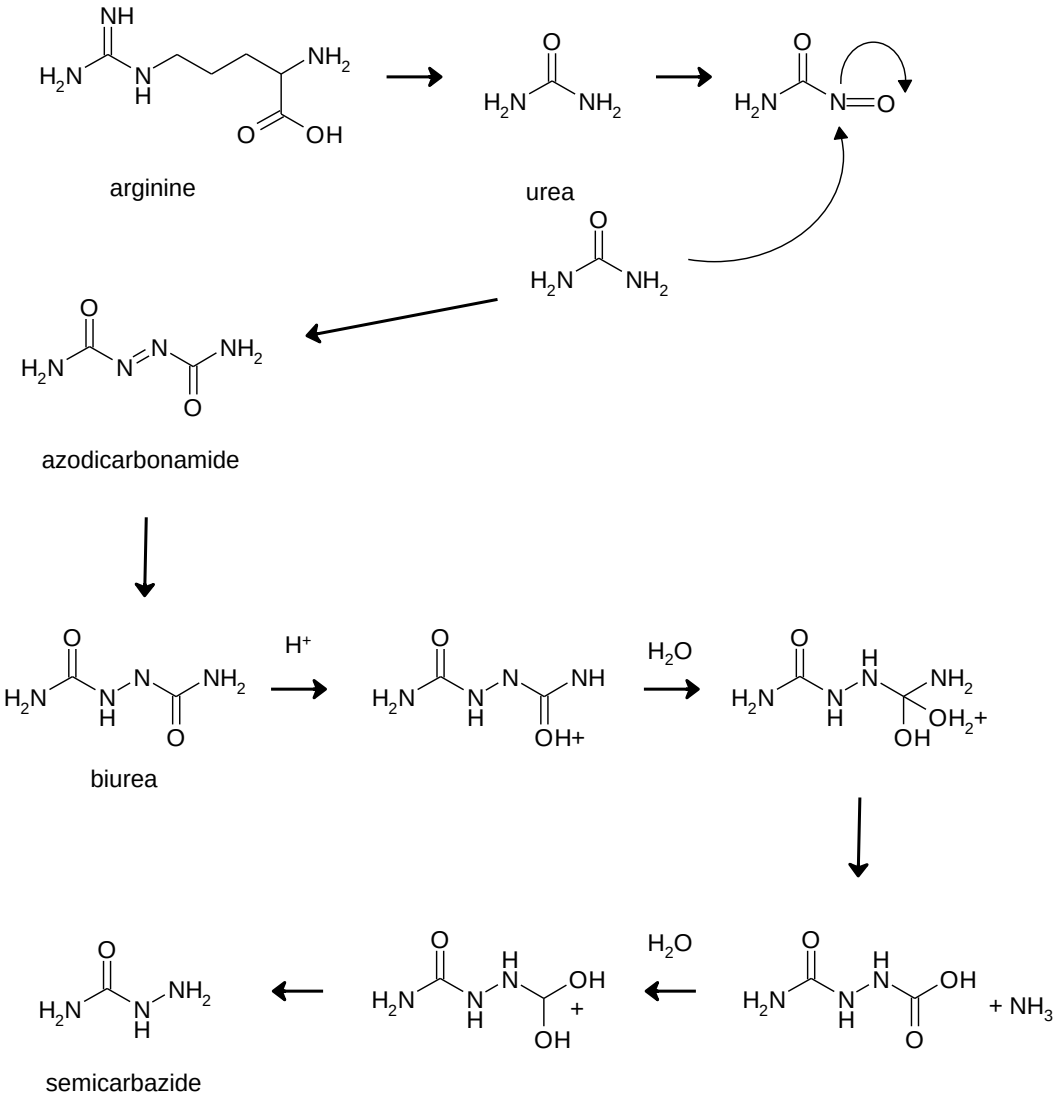


Figure 4 Formation of SEM from arginine.

Arginine in Calluna, bees, honey and nectar

There are no reports of the levels or confirmation of the occurrence of citrulline, histidine, creatine, creatinine, urea or other amides and isocyanates in heather plants, however it can be assumed that some of these compounds will be present.

Honey is produced from nectar, which is an aqueous solution of sugars, amino acids, proteins, lipids, minerals, and other components produced by flowers. Bees secrete fluids containing enzymes into the nectar that break down polysaccharides into monosaccharides.

The bees help the nectar to ripen by evaporating its water by fanning the honeycomb with their wings.

Amino acids are only present in small quantities in nectar, typically 0.002 to 4.8% of the total solids. This is considered to be too low for the dietary needs of honeybees.

The composition of honey is affected by the nectar sources. Several studies of the amino acid content of honeys have shown that arginine is present, but levels have not been found to be particularly elevated in heather honey. Certain amino acids are synthesized by the bees and are common to many types of honey (Gonzalez Paramas *et al.* 2006); others are derived from pollen, nectar, and honeydew. Pollen is by far the most important source of proteins and free amino acids for bees (Gonzalez Paramas *et al.* 2006).

Composition of heather

Heather (*Calluna*) is distributed in the Northern and Western countries of Europe, where it represents one of the most important resources for honey production in the late summer (Persano Oddo and Piro 2004). Heathland in Europe comprises a mixture of plants. The plant species from which the affected honey was produced in 2010 were partially identified for two samples by pollen analysis (*Erica* was not included) in the analysis which showed that *Calluna pollen* comprised 62% of the total in each, with *Trifolium* contributing 21 % of the total to one sample and *Brassica* contributing 15 % of the total to the other. Levels of other plant pollens were much lower. The heather plant in England and Scotland is generally assumed to be *Calluna vulgaris*, although *Erica* is a similar genus associated with heathland and is often described as “heather” on the continent and bell heather in Scotland. Serra Bonvehi and Grandanos Tarrés (1993) reported that the proportion of *Calluna* pollen in 30 Spanish heather honeys was between 10 and 33% and that the proportion of *Erica* pollen in these honeys was more than 10%. The English/Scottish common names "heath" and "heather" are shared by some closely related genera of similar appearance. *Calluna* generally flowers in the summer or autumn and *Erica* in the winter or spring. *Calluna* is frequently colonised by fungi which affect the nitrogen content of the plants and soil (van der Wal *et al.* 2009) which increase the nitrogen content of its leaves and shoots, although the details of the nitrogen’s biochemistry are not available.

No major reviews or publications were found that dealt with the chemical composition of heather (*Calluna* or *Erica*) other than the proximate levels of nitrogen, etc.

However a single publication (Parveen *et al.* 2007) concerning the metabolites found in sheep feeding on *Calluna* heather found the compound 2-imino-4(5*H*)-thiazolone in experimental dietary mixtures comprising hill grass (*Molina caerula*) and *Calluna vulgaris* in various ratios. The thiazolone was reported to be dependent on the amount of *Calluna* in the

mixture. It was measured by gas chromatography with time-of-flight mass spectrometry, which provides a very accurate molecular weight and empirical formula for analytes but not full library-searchable spectra.

The structure of 2-imino-4(5*H*)-thiazolone is given in Figure 5. The compound is unreported elsewhere and is assumed to have been identified as a structural prediction by the mass spectrometer software. It contains some elements of the SEM skeleton.

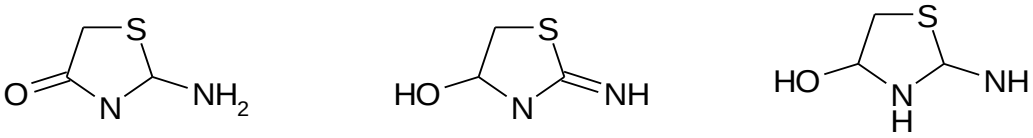


Figure 5 Structures of isomers of 2-imino-4(5*H*)-thiazolone

A further important possibility is contamination of the heather by urine from sheep. *Calluna* is a low-growing plant and therefore susceptible to contamination with urine, which contains a number of purine derivatives such as urea and creatinine that are known precursors of SEM.

Composition of heather honey

The chemical composition of honey of all types has most often been studied as part of research aimed at identifying floral markers for proof of provenance. The literature obtained is restricted entirely to the composition of the major components such as amino acids and phenols (Ferreres *et al.* 1996), carbohydrates (Sanz *et al.* 2004. de la Fuente *et al.* 2011.) and to the volatile compounds (de la Fuente *et al.* 2005).

The identification of chemicals unique to heather honey required searches of the composition of several other honeys. Data for other honeys were in any case frequently included with those for heather honeys. A list of the chemical compounds found in honey is given in the appendix. The list is not restricted to heather honey to allow comparison of heather honeys with other types. The chemicals listed for all honeys do not contain known or obviously likely SEM precursors other than the amino acid arginine.

Compounds identified as prospective natural precursors for SEM formation other than arginine (citrulline, histidine, creatinine, and urea) have not been reported in any honey, although they are widespread in animal and biological systems and may be present to some degree in *Calluna* and/or its honey.

Amino acids in honey

Heather honey is characterised by a high total protein content (Serra Bonvehí and Granados Tarrés, 1993). Data from the few publications that report the amino acid content of heather

honey are given in Table 1 and Table 2. Table 1 presents quantitative data for *Calluna* honey from Janiszewska *et al.* (2012), Davies and Harris (1982), and Rebane and Herodes (2008). The levels of arginine range from 0.4 to 9.8 mg/100g, the latter being the average of about 20 honey samples. Also reported in Table 1 are quantitative data for honey from *Erica arborea* and *Erica multiflora* from Bossi and Battaglini (1978). This includes the only data of the levels of protein-bound amino acids in honey, albeit from single samples. The arginine content of the *E. arborea* sample was similar to that of *Calluna*, but that of *E. multiflora* was much higher at about 70 mg/100g. Protein-bound arginine was present at similar or higher levels than free arginine.

Table 2 shows the percentage of each amino acid in the same samples with additional data for *Calluna* from Gonzalez Paramas *et al.* (2006). Arginine ranged from about 1 to 1.5% of the total amino acid content of *Calluna*, and 0.5 % of *E. arborea* but 8% of *E. multiflora*.

Reports for the amino acid content of other honeys from several sources show a wide variation in results for the individual amino acids and for the total weight of amino acids, which vary with country and species (Davies 1975, 1976, Battaglini 1978, Baker and Baker (1983a, 1983b, 1983c), Pirini *et al.* 1992, Conte *et al.* 1998, Mondal *et al.* 1998, Cometto *et al.* 2003, Hermosin *et al.* 2003, Gonzalez Paramas *et al.* 2006, Perez *et al.* 2007, Qamer *et al.* 2007, Pereira *et al.* 2008, Rebane and Herodes 2008, Rebane and Herodes 2010, Carratu *et al.* 2011, and Janiszewska *et al.* 2012).. The proportion of arginine in non-heather or non-specified honeys ranged from about 1 to 3.5% of the total amino acids, indicating that heather honeys do not normally contain a higher proportion of arginine than other honeys.

Amino acids in propolis and pollen

Propolis has only a very low amino acid content (dos Santos Pereira *et al.* 2003) and has no other likely SEM precursors, thus it is not considered further.

Pollen is another potential source of amino acids that that might act as SEM precursors but only limited data were found on the composition of pollen, none of which included pollen from *Calluna* (Gilliam *et al.* 1980, Krizo and Liska 1987, Loper and Cohen 1987, Serra Bonvehi and Grandanos Tarrés 1993, Iglesias *et al.* 2004, Szczesna 2006a, Szczesna 2006b, Mondal *et al.* 2009). Solberg and Remedios (1980, cited by Szczesna 2006b) reported that levels of arginine were high in pollen. The free amino acid composition of pollen from flowering plants showed pronounced homology within and between families. The major amino acids found in angiosperm pollen studied by Mondal *et al.* (2009) were amino-n-butyric acid, aspartic acid, glutamic acid, methionine, phenylalanine and proline. The other major amino acids present in free form included arginine, cysteine, glutamic acid, glycine, isoleucine, leucine, ornithine, tryptophan and tyrosine. It has been shown that the amounts

of arginine and some other amino acids (phenylalanine, proline, and aspartic acid) in pollen vary throughout the year (Kauffeld 1980).

Honey bees feed mostly on pollen, and to a lesser extent on nectar and honey, but it is usually stored in the cells of the combs for a period before consumption. Bees appear to secrete substances that might affect the composition of comb-stored pollen (Haydak 1958), but the changes that occur are not well understood. Bee larvae may consume up to 2 mg of pollen each per day. Most of the pollen (75% of the grains) is completely digested and only about 2% excreted undigested. However the bee larvae obtain less than 5% of their required protein from pollen (Babendreier *et al.* 2004). Stored pollen does not differ significantly after 7, 21 and 42 days storage in comb cells, either in the content of the individual amino acids or the total amino acids (Standifer *et al.* 1980).

However studies of the amino acid content of dew showed some interesting changes in environmental levels of amino acids probably derived from pollen (Scheller 2001). In dew samples collected from two sites in Germany from June 1996 to June 1997 and analysed for free and protein-bound amino acids the quantity of amino acids rose during the flowering season, indicating a plant source, although the plants were not identified. The pollen content was high at the beginning of June 1996 and in May 1997 with total amino acid concentrations were typically 50-400 micromol/litre (µmol/L), with one sample reaching 922 µmol/L. During the other times when pollen was lower the amino acid concentration in dew was 8-164 µmol/L. In June 1996 the amino acid content was predominantly arginine, proline and glutamine/glutamate, and again in March 1997 the arginine and proline levels were extremely high. The content of glycine and serine decreased during this time. The effect occurred in both 1996 and 1997 over several days at both sites at any one time. This is a good indication that within the same plant populations sudden and short-term high increases in arginine content are possible.

Other compounds in heather and other honeys

Chemicals reported present in many European honeys are listed in the Appendix. The list is based on data from the recent survey by Plutowska *et al.* (2011) who analysed Polish honeys from rape, acacia, linden, buckwheat, heather, polyfloral and honey-dew and reported over 300 compounds, of which 178 were identified. Additional data comes from Baker and Baker (1983a, 1983b, 1983c), Speer and Montag (1984, 1987), Steeg and Montag (1987), Steeg and Montag (1988a,b), Blank *et al.* (1989), Tan *et al.,* (1989a, 1989b), Hausler and Montag, (1990, 1991), Bouseta *et al.* (1992), Iason *et al.* (1993), Ferreres *et al.* (1994), Martos *et al.* (1997), Guyot *et al.* (1999), Tomas-Barberan *et al.*,(2001), Perez *et al.* (2002) Ammar *et al.* (2004), de la Fuente *et al.* (2005), Gonzalez Paramas *et al.* 2006, 2007,

Wolski *et al.* (2006), Soria *et al.* (2003, 2004, 2009), Rieger *et al.* (2008), and Radovic *et al.* (2001).

Guyot *et al.* (1999) reviewed studies of volatile characteristic marker compounds for heather honeys (particularly from Poland). They identified 48 aroma compounds in extracts of honey derived from the heather plants *Calluna vulgaris* and *Erica arborea*. Most were also constituents of honeys of other origins. A few compounds were distinctive on account of their presence in *Calluna* and *Erica* at levels significantly different from those recorded in the other honeys. The compounds identified as distinctive for Polish heather honey were benzoic acid, phenylacetic acid, dehydrovomifoliol (4-hydroxy-3,5,5-trimethyl-4-(3-oxo-1-butenyl)-2-cyclohexen-1-one) and isophorone. Heather honeys had relatively high levels of benzoic acid and phenylacetic acid. Dehydrovomifoliol is a growth phytohormone derivative of abscisic acid. Others (Radovic *et al.* 2001) found no volatile substances characteristic of heather honey in samples of various botanic origins including heather.

Soria (2008) reported that dimethylphenylethanone was a major component of the volatiles of heather honey (13% of the total volatiles). The compound was absent in honeys from orange blossom, lavender, rosemary and multiflower honeys but also present in honeydew honey at 13% of the volatiles.

In a survey of 395 tropical and temperate floral nectars, Baker and Baker (1983a, 1983b, 1983c) found a variety of amino acids and other compounds including lipids, organic acids and minerals. Honeys from *Ericaceae* family plants obtained in France, Greece and Italy had high levels of aromatic carboxylic acids. These included cinnamic acid, which was present at 0.9 to 3.3 mg/kg, higher than reported present in German and Scottish heather honeys (< 2 mg/kg) by Steeg and Montag (1987). It was absent in 11 other honeys investigated. Benzoic acid was present in heather honeys at concentrations of 2 to 64 mg/kg, higher than in non-heather samples. Similar values (5-216 mg/kg) were reported for French, German, and Scottish heather honeys by Speer and Montag (1984) and Steeg and Montag (1988b). *Calluna vulgaris* honeys had significant levels of 4-(3-oxobut-1-enylidene)-3,5,5-trimethyl-cyclohex-2-en-l-one, greater than found in *Erica* honeys. The honeys also contained isophorone (3,5,5-trimethylcyclohex-2-en-l-one) at high level (up to 1453 µg/kg. These compounds are not unique to heather honeys.

Phenylacetic acid is found exclusively in *Calluna vulgaris* honeys, at concentrations varying from 0.6 to 977 mg/kg (Speer and Montag, 1984, 1987). Other compounds possibly unique to both *Erica* and *Calluna* are dehydrovomifoliol (4-hydroxy-4-[3-oxo-l-butenyl]-3,5,5-trimethylcyclohex-2-en-l-one) and 4-(3-oxo-l-butynyl)-3,5,5-trimethylcyclohex-2-en-l-one (Guyot *et al.* 1999; Hausler and Montag, 1991; Tan *et al.,* 1989a) The related 4-(3-oxo-l-

butynyl)-3,5,5-trimethylcyclohex-2-en-l-one has been measured in *Calluna vulgaris* honeys from New Zealand (Tan *et al.,* 1989a).

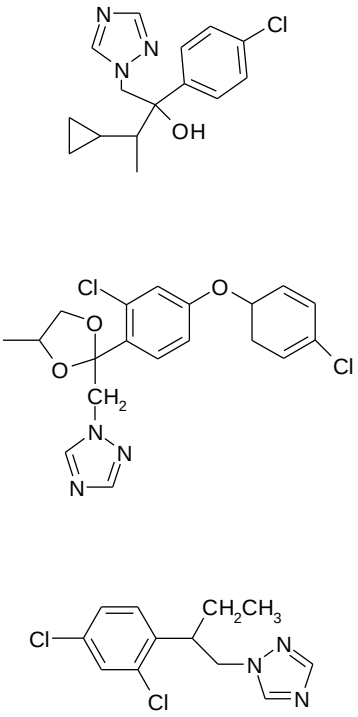
The gallic acid dimer ellagic acid has been confirmed as a marker of *Calluna* honey (Tomas-Barberan *et al.*, 2001), with *Erica* honey being characterized by the presence of hydroxybenzoic, syringic, *O*-coumaric and ellagic acids.

Heather honeys from both *Calluna* and *Erica* contain 4-methoxybenzaldehyde, which is absent from other honeys, at concentrations of 0.07-2.5 mg/kg (Guyot *et al.* 1999; Hausler and Montag 1990; Tan *et al.* 1989b; Blank *et al.* 1989). *Erica* alone has been found to contain 4-methoxybenzoic acid and 4-hydroxy-3-methoxybenzoate methyl ester (Steeg and Montag, 1988a), which was not detected in any of 128 other samples as a specific marker of *Erica arborea* honeys (13-1350 mg/kg).

No compound in these reasonably extensive lists other than the amino acids listed separately has elements of the SEM molecule. It is very unlikely that other compounds would give a response that could be misinterpreted as SEM in the LC-MS/MS analytical system.

Potential SEM precursors present through environmental contamination.

Several agricultural chemicals contain compounds that have the potential to be converted into SEM, and very many organic compounds are frequent or irregular contaminants of the environment. Contaminants of honey were described by Bogdanov (2006) as provided in Table 1, but of the rather few compounds listed, only three have an N-N-C-N component similar to SEM and might lead to its formation. These are the water-based wood preservatives cyproconazole, difenoconazole, and penconazole, the structures of which are given in Figure 6.



Top: Cyproconazole, centre: Difenocnazole, bottom: Penconazole.

Figure 6 Fungicides with partial SEM structure detected as contaminants in honey.

Another possible source of SEM contamination is the transfer of nitrofurans or other SEM precursors from environmental sources into the honey by bees. In addition to collecting pollen and nectar bees forage for considerable quantities of water and might pick up nitrofurans or other precursors through collecting water contaminated by chemical application elsewhere, or from dew containing arginine.

CONCLUSIONS

Possible reasons for the positive finding of SEM in heather honey

1. SEM was present in the heather honey through transfer from Calluna plants.

It is possible that SEM is a natural constituent of the *Calluna* plant, or parts of the plants such as nectar or pollen. There is no published evidence of this, but until now there has been no reason for this potential occurrence to be studied.

2. Natural SEM precursors were present in the heather honey through transfer from Calluna plants.

The most likely natural precursor of SEM in heather honey is the amino acid arginine, although other nitrogenous compounds (including but not limited to ornithine, citrulline, creatine, and creatinine) might be partly or wholly responsible. The evidence for this is the demonstrated formation of SEM from the arginine and citrulline in some foods, and from arginine alone in eggs and whey on warm storage.

Further support is given by the fact that high levels of arginine occur in certain shellfish that have been shown to contain SEM naturally, and that there is evidence of short-term surges of arginine in the environment (in dew) that are likely to be due to pollen.

It has been shown that SEM can be formed under relatively mild conditions from arginine, that the arginine content of some, maybe several, unidentified plants increases rapidly at certain times of the year, and that bees probably digest proteins that can contain arginine, with the production of urea. Higher than ambient temperatures in the hive might promote chemical reactions of arginine.

Thus it is possible that since SEM can be formed from arginine on incubation *in vitro* it could also be formed either in the *Calluna* plant or in the bee, or in the honey in the hive. The infrequent finding of SEM in honey from untreated hives might be due to the fact that sparse sampling regimes are likely to miss brief incidences of high-arginine pollen production. Alternatively, *Calluna* could be contaminated with urine from sheep, and the SEM precursors in the urine carried into honey.

3. Natural or non-natural SEM precursors were collected by the bees from other sources.

This is possible but unlikely. The limited pollen analysis shows that the majority of the plants visited to produce the honey were of *Calluna*. The most probable alternative source would be through water consumed by the bees and the chemical composition of this would depend on local factors.

4. The bees' metabolic processes can produce SEM from precursors that are present in heather.

It is possible that bees could produce SEM on their bodies and pass it into honey. This aspect of bees' metabolism has not been studied with respect to SEM, but it would still require a precursor derived from heather.

5. Natural or non-natural SEM precursors were present in the heather honey through environmental contamination.

The possibility that the source of SEM contamination was environmental contamination or the transfer of nitrofurans or other SEM precursors (such as arginine in dew) from environmental sources into the honey by bees is slight, but would produce the contamination limited in area and time as was measured. It is also possible that unidentified potential environmental precursors exist.

6. The honey samples were contaminated through packaging.

It is well established that SEM can be formed from azodicarbonamide (ADC), a blowing agent used to foam the plastic sealing gaskets in metal lids on jars or bottles and that SEM produced from ADC can migrate into foods. However in the current case the heather honey samples were taken directly from the hives and the possibility of contact with ADC, which is no longer approved for use in food contact materials, can be excluded.

7. The honey samples were contaminated with SEM derived from bleaching activities.

SEM is formed as a reaction product from the treatment of some foods, notably carrageenan egg white powder, with hypochlorite. The hives from which honey with SEM was obtained were brand new plastic hives, which would not have required disinfection. In any case bleach is not routinely used for disinfection of hives as it would affect the honey. Therefore the use of bleach would be a very unlikely source of SEM.

8. SEM was present in wax added to the hives

Beekeepers provide a beeswax-foundation for their bees that is based on recycled beeswax from old comb. In the event that this beeswax was derived from colonies treated with nitrofurazones an accumulation might occur and lead to residues in the honey of the hive receiving the foundation. The extent of contamination by this potential transfer has however been shown to be limited (Bogdanov et al. 1998).

9. The test results were falsely positive.

The LC-MS/MS analytical method was applied by a laboratory accredited for this test. It is highly unlikely that false positive results were obtained.

It is somewhat strange that positive findings of SEM were reported in 2010 and possibly not in previous years, and that natural SEM was found predominantly in Scotland. This could be due to increased frequency of testing or to improvement in the analytical methods, or to some external event that caused SEM or its precursor to be produced in that single area at that time. However SEM residues have also recently been confirmed at about 1 µg/kg in two samples of New Zealand wild forest honey analysed in the UK by an accredited laboratory.

10. Nitrofurans were used on the hives producing the honey.

Few publications describe the use of nitrofurans in beekeeping but it has been reported that furazolidone is the main nitrofuran antibiotic administered to treat bacterial diseases of bees (Khong *et al.* 2004). Furazolidone or its metabolites can be detected in honey over 300 days after dosing (CSL 2006). The hives for which positive results for SEM were reported were believed to be new, and SEM was not reported in honey taken before or after the heather honey that contained it.

The findings of SEM followed major outbreaks of both European foul brood (EFB) and American foul brood (AFB) in Scotland in the autumn of 2009 (Defra 2012). EFB was present in about 10% of 3000 Scottish hives monitored in 2009, much higher than was reported in both earlier and later years. AFB was present in about 5% of hives monitored in 2009, more than ten times the level for 2010 and fifty times that for 2011 (Scottish Beekeepers Association 2012). However the antibiotic oxytetracycline was used against EFB at this time and the hives were under increased scrutiny by Bee Inspectors and the Veterinary Medicines Directorate VMD. Use of a different (and illegal) antibiotic would bring little benefit and much risk to the beekeepers.

11. A different non-natural class of SEM precursor was applied to the hives.

This is very unlikely as only a very limited number of compounds are applied to hives and no candidate precursors have been identified.

Recommendations for potential techniques and further work.

1. The report of SEM in heather honey and its restricted occurrence to short time periods should be verified. Samples should ideally be taken from other areas of heather growth.

2. The nitrofuran analysis should be carried out using more than one technique, one of which should use an alternative marker. Determination of 5-nitro-2-furaldehyde has been used for detecting nitrofuran antibiotics at low µg/kg levels (Ritchie *et al.* 1977). However this is no more specific than SEM and it has two major disadvantages in that the nitrofurans are believed to lose the nitro group early in their complex metabolism, and it too is also a small, simple molecule that could potentially arise from natural sources.

3. The analysis of protein isolated from honey could be analysed by a variant of the method for nitrofurans that enables measurement of the tissue-bound SEM. This method was devised under the EU project QLK1-CT-1999-00142 (FoodBRAND 2000). It could indicate whether the SEM in honey was protein associated (suggestive of nitrofurazone use) or otherwise, and avoid positive results derived from contaminating sources of SEM from ADC, bleach, etc.

4. To help study the likely natural formation from arginine in heather pollen, samples of pollen and dew from *Calluna* and *Erica* plants (and other plants in the vicinity) should be collected and analysed. Ideally such samples should be collected in the bee foraging areas at regular intervals before, during, and after the flowering period.

5. The possible contribution of sheep's urine to SEM formation from heather should be studied.

6. Analysis of heather honey and non-heather sources as control honeys should include a profile and quantitative measure of the amino acid composition. Ideally this should be carried out in a manner that shows a profile of amino acid composition over several months.

7. Samples of bees and bee parts, particularly the cuticle should be analysed for SEM and arginine.

8. Laboratory studies should investigate the *in-vitro* formation of SEM from the naturally occurring precursor compounds identified above.

Action plan for potential further work.

Further work to meet the recommendations above can best be divided into field based and laboratory based projects as field studies would indicate the real situation and laboratory work would provide clear evidence of the potential for SEM formation, and allow characterisation and understanding of the mechanisms and the effects of environmental influences. Under suitable circumstances simulated 'field' work could be conducted in the laboratory.

Field studies

1. Monitoring SEM in heather honey.

The occurrence of SEM in honey should be monitored. However to take account of localised and occasional occurrences the sampling period ideally needs to be long and the frequency of sampling high. This is probably the only way to get an accurate picture of the real life situation. Initially this experiment would best be performed in Scotland.

The flowering times of *Calluna* vary depending on climate but in Scotland typically range from June to August. Heather honey samples should be collected from a number of hives from June to September or October and analysed for SEM. Additional sampling could be carried out for *Erica*. Honey analysis can be conducted in relatively large batches, typically monthly, or even at the end of the sampling period. It will be important to take regular and frequent samples from a number of locations and the sampling might be the major cost. At a minimum, sampling two honeys from each of five hives in each of just five heather locations weekly for five months would provide 1000 samples for analysis. Additional control samples from non-heather areas would be required. To reduce sampling costs the samples would ideally be provided by the beekeepers provided that the sampling procedures and the sample containers were provided to them to ensure the reliability of the samples provided.

Benefits: An accurate picture would be obtained of natural SEM occurrence, but no information on its formation mechanisms.

Costs: The cost of analysis of these samples and management and reporting would be relatively low but the cost of sampling by bee inspectors would be considerable.

2. Monitoring SEM and arginine in *Calluna* and *Erica* heather.

The potential presence of SEM or its precursors in *Calluna* and *Erica* heather will confirm *Calluna* or *Erica* as the source of SEM in heather honey. Once again, as arginine production is suspected to be occasional, frequent and regular sampling would be required. However in this instance the sampling would require dedicated local staff, possibly bee inspectors, at more considerable cost. Samples should comprise *Calluna* and *Erica* pollen, whole plants from sheep-grazed and sheep-free areas, and dew if available.

Benefits: Confirmation of *Calluna* or *Erica* as the source of SEM in heather honey and possible identification of formation routes.

Costs: Normal commercial costs of analysis for SEM and amino acids would apply but the costs of sample collection in the wild might be prohibitive.

Laboratory studies

1. Comparison of analytical methods for SEM.

This would verify that there might be a better marker of nitrofurazone abuse and confirm a natural source of SEM in any SEM-positive honeys encountered. Given that the levels of SEM in the affected honeys was very low (0.6 and 1.8 µg/kg) and that any alternative metabolites could be less abundant (lower concentrations) than SEM, the cost of method development and consequent testing could be prohibitive. However, the analysis of tissue-bound SEM in honey by a variant of the method for nitrofurans would be useful.

Benefits: Provision of alternative methods would distinguish between natural and nitrofurazone sources of SEM.

Costs: Method comparisons and in-house validation would be relatively expensive and be quite uncertain of success.

2. Determination of the arginine and SEM content of *Calluna* and *Erica* pollen and nectar, and whole plants.

Plants of *Calluna* and *Erica* could be grown at a suitable site and their pollen and plant tissues analysed frequently for the amino acid profile and content of arginine and possibly other potential SEM precursors such as creatinine. *Calluna* and *Erica* can be treated with sheep urine to investigate this source. Cultivation at the analysis site would effectively remove the bulk of the sampling costs for field studies. The pollen and plants could be analysed for SEM to confirm whether or not it is produced in the plant.

Benefits: Confirmation of peaks in arginine production through the flowering period, identification of precursors and the source of SEM, greater confidence on account of high sample numbers, reduced costs of sampling.

Costs: these would be moderate, depending on the scale of the experiments.

3. Determination of the SEM and arginine content of heather honey and control honeys.

A survey of market samples of heather honey for their content of SEM would improve the scope of the hive sampling and allow an opportunity to study the profile of the amino acid composition and specifically arginine content. This would involve analysis of retail samples of heather and non-heather control samples.

Benefits: Confirmation of arginine as precursor without reliance on beekeepers providing samples.

Costs: The usual moderate costs of food sampling and analysis would apply.

4. Investigate the *in-vitro* formation of SEM from the naturally occurring precursor compounds identified above.

Laboratory experiments could quickly confirm whether SEM formed from nitrogenous precursors including arginine and creatinine. The experiments would include heating over a range of times and temperatures and the catalytic effect of precursor mixtures, and the effect of the presence of a honey matrix.

Benefits: Identification of precursors and conditions needed for natural formation of SEM.

Costs: Relatively brief laboratory experiments at low cost.

5. Investigate the *in-vivo* formation of SEM from the naturally occurring precursors by bees.

Feeding studies involving bees given arginine and other SEM precursors would show whether or not bees play a role in the formation of SEM in honey. Bees can be fed a range of precursor compounds singly or in combination and SEM and precursors measured in the bees and hive products.

Samples of bees and bee parts, e.g. cuticle will be analysed for SEM and arginine.

The honey produced from such bees could be analysed regularly to identify any compositional changes on maturation.

Benefits: More detailed information on formation in the hive.

Costs: Moderate costs typical of a research project operated over 9-12 months.

Table 1 Amino acid composition of *Erica* and *Calluna* honeys (mg/100 g)

Source	A	A	A	A	B	C	D
No. samples	1	1	1	1	3	5	19
Honey free/protein	*E. arb* free	*E. multi* free	*E. arb* protein	*E. multi* protein	*Calluna* free	*Calluna* free	*Calluna* free
α-Aminoadipic					0.13		
α-Aminobutyric					0.11		
β-Aminobutyric					0.57		
Alanine	1.66	1.64	8.14	10.19	0.98	1.45	13.80
β-Alanine					0.83		6.90
Arginine	**0.68**	**68.33**	**14.76**	**15.49**	**0.36**	**1.23**	**9.80**
Asparagine	2.05	2.73	41.00	40.82	0.58	2.75	7.90
Aspartic acid					0.69		12.2
Cysteine	0.86	17.20	0.52	2.29			
GABA[1]					0.37		4.10
Glutamic acid	2.06	6.00	24.90	26.00	0.91	6.68	17.30
Glutamine					0.76		11.20
Glycine	0.52	0.87	7.33	12.83	0.41	0.53	5.50
Histidine	0.67	9.33	0.40	1.33	0.26	0.58	3.80
HYP[2]	0.66	0.26	1.26	3.51			
HSER[3]							
Isoleucine	0.89	9.03	14.34	13.31	0.41	0.51	5.50
Leucine	0.48	0.64	22.03	22.72	0.53	0.48	7.00
Lysine	3.36	2.42	9.82	12.59	0.82	2.57	12.00
Methionine	0.86	0.29	0.77	2.87	0.10		
Ornithine					0.05		
Phenylalanine	54.66	699.73	13.94	23.69	1.60	5.67	19.70
Proline	64.96	48.43	9.98	17.06	28.41	68.16	487.40
Serine	1.83	4.67	1.00	31.11	0.67	1.30	9.90
Taurine							
Threonine	0.17	0.45	1.21	21.55	0.55	2.66	5.30
Tryptophan	0.30						
Tyrosine	3.10	1.32	1.79	15.24	0.50	8.11	8.70
Valine	1.24	1.76	16.43	13.64	0.68	1.06	8.00

E. arb = Erica arborea

E. multi = Erica multiflora

1 GABA = γ-aminobutyric acid.

2 As published = hydroxyproline?

3 As published = hydroxyserine?

A =Bossi and Battaglini 1978; B = Janiszewska *et al.* 2012; C = Davies and Harris 1982; D = Rebane and Herodes 2008.

Table 2 Amino acid % composition of *Erica* and *Calluna* honeys

Source	A	A	A	A	B	C	D	E
No. samples	1	1	1	1	3	5	19	1
Honey free/protein	*E. arb* free	*E. multi* free	*E. arb* protein	*E. multi* protein	*Calluna* free	*Calluna* free	*Calluna* free	*Calluna* free
α-Aminoadipic								0.6
α-Aminobutyric								
β-Aminobutyric								
Alanine	1.2	0.2	4.3	3.6	2.4	1.4	2.1	2.2
β-Alanine					2.0		1.1	
Arginine	**0.5**	**7.8**	**7.8**	**5.4**	**0.9**	**1.2**	**1.5**	**1.3**
Asparagine	1.5	0.3	21.6	14.3	1.4	2.7	1.2	3.1
Aspartic acid					1.7		1.9	3
Cysteine	0.6	2.0	0.3	0.8				
GABA[1]					0.9		0.6	3.4
Glutamic acid	1.5	0.7	13.1	9.1	2.2	6.4	2.6	
Glutamine					1.8		1.7	1.7
Glycine	0.4	0.1	3.9	4.5	1.0	0.5	0.8	0.7
Histidine	0.5	1.1	0.2	0.5	0.6	0.6	0.6	0.6
HYP[2]	0.5		0.7	1.2				
HSER[3]								0.5
Isoleucine	0.6	1.0	7.6	4.6	1.0	0.5	0.8	1.1
Leucine	0.3	0.1	11.6	7.9	1.3	0.5	1.1	0.5
Lysine	2.4	0.3	5.2	4.4	2.0	2.5	1.8	1
Methionine	0.6		0.4	1.0	0.2			1.5
Ornithine					0.1			3.7
Phenylalanine	38.8	80.0	7.4	8.3	3.9	5.5	3.0	12.9
Proline	46.1	5.5	5.3	6.0	68.8	65.7	74.3	49.6
Serine	1.3	0.5	0.5	10.9	1.6	1.2	1.5	1.4
Taurine								4.1
Threonine	0.1	0.1	0.6	7.5	1.3	2.6	0.8	0.8
Tryptophan	0.2							4.4
Tyrosine	2.2	0.2	0.9	5.3	1.2	7.8	1.3	1.1
Valine	0.9	0.2	8.7	4.8	1.7	1.0	1.2	0.5

1 GABA = γ-Aminobutyric acid.

2 As published = Hydroxyproline?

3 As published = Hydroxyserine?

A =Bossi and Battaglini 1978; B = Janiszewska *et al.* 2012; C= Davies and Harris 1981; D =Rebane and Herodes 2008; E = Gonzalez Paramas *et al.* 2006.

Table 3 Environmental contaminants found in honey

Organic contaminants	Polychlorinated biphenyls Polyaromatic hydrocarbons
Pesticides used in agriculture	Organochlorines Organophosphorus pesticides Carbamates: pesticides
Fungicides	Asulam Captan Carbendazim Cyproconazol Difenoconazole Dithianon Iprodione Methyl thiophanate Penconazole Pyrifenox Vinclozolin
CONTAMINANTS FROM BEEKEEPING	
Acaricides	Amitraz Coumaphos Cymiazole Flumethrin Fluvalinate Formic acid Lactic acid Oxalic acid Thymol
Antibiotics	3-Amino-2-oxazolidinone Chloramphenicol Nitrofuranes Penicillins Streptomycin Sulfonamides Tetracylines Tylosin
Other substances	Naphthalene Para-dichlorobenzene Pentachlorophenol Phenol

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