Q&A on the production of nootkatone (an ingredient of grapefruit flavour)

With Jason King, CEO Oxford Biotrans
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1. What is nootkatone?

Nootkatone is a fine chemical from grapefruit. It is a key contributor to the fruit's unique flavour and aroma.

It is also found in other plants and may have derived from valencene, a very similar chemical that plays a role in the smell and taste of oranges. Grapefruit are a hybrid of two citrus fruit – pomelo (*Citrus maxima)* and sweet orange (*Citrus sinensis*).

Both nootkatone and valencene are plant terpenes.

2. What are terpenes?

Terpenes are highly valued, highly complex and highly diverse natural chemicals. The reactions that take place inside cells to create them are some of the most complex in biology.

They are volatile compounds so are easily released, making them easy to smell and taste. They are released by trees to seed clouds in warm weather and by plants to provide defence against insects and disease. The hoppy taste and smell of beer is from terpenes and they are widely used in the food and perfume industries. They also have anticancer, antimicrobial and anti-inflammatory properties that make them valued in agriculture, medicine and nutrition. For example, vitamin A is a terpene.

They are produced by plants in such small amounts that extracting them is difficult and expensive. Scientists all over the world are studying cheaper and more sustainable ways to make them.

3. What is so special about grapefruit flavour?

The Latin name for grapefruit is Citrus paradisi and the fresh, tangy flavour makes it valued not only as fresh fruit and juice, but in a wide range of drinks, puddings and confectionery. It is one of the most expensive flavour ingredients in the world.

As Martha Stewart says:
"If lemons are sour and oranges sweet, grapefruit picks up the slack in between. With its signature zing and slightly bitter finish, it adds bright complexity to sweet and savoury dishes alike."

4. Why not just source grapefruit flavour from grapefruits?

It takes around 400,000kg of grapefruit to extract 1kg of nootkatone. Supply of grapefruits is affected by disease, hurricanes and falling demand for the fresh fruit and juice. During hurricane years, nootkatone can be impossible to source.

While grapefruit production has been in decline over the last 15 years, demand for the flavour remains high.

An alternative is to take one of the compounds responsible for the flavour of orange, valencene, and to chemically convert it. Or valencene can be derived from a genetically-engineered yeast and then chemically oxidised. But the product cannot be labelled as natural. Novel, biological (and chemical-free) routes for a natural ingredient have been sought.

5. What's wrong with synthetic nootkatone?

The chemical oxidation of valencene to make nootkatone is dependent on some undesirable heavy metals and peroxides, such as chromium, manganese, tert-butyl chromate and tert-butyl peracetate. Chemists have been trying to design a more environmentally sustainable method for decades.

This first application of the Oxford Biotrans technology is just the start of the greening of chemical production. The scientists are developing processes for making a wide variety of chemicals in a more efficient and environmentally friendly way to conventional chemistry.

6. Could the new technology deprive grapefruit farmers of a market?

Grapefruit farming is driven by demand for the fresh fruit and the juice. The oil and oil extracts are by-products. A new sustainable source of natural nootkatone could actually lead to new grapefruit-flavoured products coming onto the market and in turn reignite interest in juice and fresh fruit consumption.

There are also many other demands for nootkatone besides as a flavour. For example, in the perfume industry. Research has shown the compound has potential as a natural insect repellent.

7. How does the new technology work?

Scientists from the University of Oxford and spin-out company Oxford Biotrans have identified a way for an enzyme called cytochrome p450 to convert nootkatone from valencene.

Out of the lab, cytochrome p450 adds oxygen atoms to molecules. Bacteria, plants and humans all make different forms of the enzyme that are able to act on different molecules. It has been called “the Swiss army knife” of enzymes because of its many uses. Each form has a unique structure.

To make a form able to add one oxygen atom to valencene and take away two hydrogen atoms, the scientists took a naturally occurring P450 from a bacterium found in many habitats, *Bacillus megaterium* and made limited but precise changes to the structure of the enzyme. The resulting enzyme is patented. See patents WO 00/31273 and WO 2009/047498.

A genetically engineered strain of the *E. coli* bacterium is used to produce this enzyme in a fermentation tank. This part of the process is carried out by a company that makes enzymes for the food industry.

The specially adapted enzyme is taken to a second location for the reaction with valencene. Valencene and the enzyme are added to a stainless steel vessel filled with water. The reaction creates nookatone, which is distilled from the mixture. The cytochrome p450 enzyme is not part of the final product.

The nootkatone can be delivered at the purity the customer wants, or blended to imbue the flavour with particular notes. 85% purity is often most desirable. It is a viscous yellow liquid.

8. From where will you source valencene?

Orange flesh and peel contain orange essential oil. Citrus oil producers source orange essential oil from juice manufacturers. They extract valencene from the oil. Even in years of poor orange harvest, valencene supply is plentiful.

The major growing regions for oranges are Brazil (50%) and (Florida 32%). Others including Mexico, India and Spain make up the remaining 18%.

9. Does the production of the enzyme fall under the definition of synthetic biology?

There is debate about the definition of synthetic biology within the scientific community. The term can be and is used to cover a very broad spectrum of activities. Mostly the term it is understood to apply to the engineering of biological systems, ‘reprogramming’ of synthetic pathways and design of ‘biological parts’ from the ground up. Cells can be turned into ‘microbiological factories’. We have not employed these approaches so, under these definitions, nothing we do would be considered synthetic biology.

What we have done is to make specific changes to a naturally-occurring enzyme – substituting a few amino acids within the entire enzyme structure. The changes are so small that they could have arisen through natural variation in the enzyme. We then produce this enzyme by fermentation as described in Q7.

Although this doesn’t fall within the understanding of synthetic biology described above, some of the much broader understandings of the term synthetic biology could encompass what we have done. So we have chosen to use this term while explaining the specifics of our technology.

In terms of regulation, the specifics of the science are assessed by regulatory authorities rather than the terminology we use. For the commercial production of enzymes by fermentation, a strictly defined regulatory framework exists. This includes regulations covering the required risk assessments, labelling requirements and traceability measures.

We also need to comply with regulation EC1332/2008 on the use and safety of enzymes used as processing aids in the production of food ingredients. In addition, EC1334/2008 lays down all requirements covering the production of flavour ingredients.

10. Is it safe to use synthetic biology in food?

In this case, the synthetic biology happens before the production of the food ingredient. Synthetic biology is used to create an enzyme which in turn is used to convert one flavour compound into another. The creation of the enzyme via synthetic biology happens in one location and the conversion of the flavour compound happens at a separate site. There are no traces of the enzyme in the final product.

A similar process is used in cheese-making.

To curdle milk, rennet was traditionally used. It contains enzymes produced in the stomach of newborn calves to help them absorb the nutrients from milk. A vegetarian alternative is to genetically engineer a bacterium to make the most important enzyme, chymosin, via fermentation. This is how most cheese is now produced. A similar process is used to make insulin, which was previously taken from the pancreas of cows and pigs.

Synthetic biology is a technology rather than a product in itself. The safety of a product depends on what it is, rather than the method used to produce it.

11. Is fermentation safe?

Fermentation is a way to convert sugar compounds into gases, acids, alcohol or other desirable products. Our bodies use it to ferment lactic acid when oxygen becomes depleted. In food production, it has been used for about 10,000 years. For example, it is used to leaven bread, to produce wine and beer, and to thicken milk to produce cheese and crème fraiche.

In food processing, a yeast or bacterium is added to the starting material (dough, grapes, milk etc). To produce cheese, the extra enzyme chymosin is also added, which itself is also created by fermentation.

Chymosin production for cheese-making is similar to the production of cytochrome p450 via fermentation with *E coli*.

12. Is this similar to Evolva’s technology to produce vanillin?

The production of nootkatone is one step further from synthetic biology compared to this method for producing vanillin.

To produce vanillin, glucose is fermented with a genetically engineered yeast strain.

To produce nootkatone, first an enzyme is produced by bacterial fermentation. Fermentation with *E coli* produces a slightly different form from the wild type enzyme.

The next stage is that the enzyme is mixed with an orange flavour compound. The enzyme converts it into a compound found naturally in grapefruit.

13. How can this be called "natural"?

The EU has the strictest regulations in the world for labelling food flavourings as natural and the UK's Food Standards Agency has also produce guidelines. It might not be possible to label an ingredient "natural" in the EU that can be labelled with the term in the US and elsewhere. The relevant regulation is EC 1334/2008 (also see Q9) and is considered as the international gold standard.

The starting materials (the enzyme cytochrome p450 and valencene) and the final product (nootkatone) are all found in nature.

Valencene occurs naturally in oranges and orange juice.

The final product, nootkatone, occurs naturally in grapefruits and grapefruit juice.

Over 21,000 forms of the enzyme cytochrome p450 exist in nature, each able to bind to and oxidise a slightly different compound (combine it with oxygen). The unusual ability of the enzyme to bind to and react with such a wide range of compounds (called "substrate promiscuity") has made it an interesting subject for study.

For example, most of the 50 or so forms of the enzyme that exist in humans are produced in the liver. They are necessary for detoxifying chemicals and for metabolising drugs. Of the thousands in plants, many create valuable defence chemicals.

The only difference with the form of the enzyme developed by Oxford Biotrans scientists is that it is able to bind to and react with valencene, a flavour compound found in oranges. The difference is so small it could have been achieved through natural selection.

14. Is the enzyme part of the final product?

No. Once the reaction of valencene with the enzyme has taken place, the nootkatone is separated from the mixture by fractional vacuum distillation. This is a way to separate compounds using their boiling point. The mixture is heated and the enzyme - a massive molecule compared to nooktatone - is simply left behind while the nootkatone is vaporised and then cooled so it can be collected.

Nevertheless, we still need to comply with regulation EC1332/2008, which not only considers safety of food enzymes that remain in a product, but also ones that are simply processing aids later separated from the final product (as in this new technology).

15. Why is only chemical synthesis called "synthetic"?

The terminology is determined by EU regulations. A chemical process, for example the use of heavy metals and peroxides to convert valencene to nootkatone, results in a product that can only be marketed as synthetic.

A method to produce a compound found in nature, that uses starting materials found in nature, and where the factory is a living organism, results in a product that can be called natural. The ingredients and the process are all biological.

16. Is this just for profit?

The quest for an alternative source of nootkatone has been driven by the need for a stable supply, a non-toxic production method, and a cheaper process.

Researchers around the world have been on separate quests. Some have found a way to produce valencene using synthetic biology (and then to chemically convert it to nootkatone). The first all-biological method for producing nootkatone is the product of UK research.

The University of Oxford researchers were publicly funded, mainly through the EPSRC. The UK is a world leader in many areas of research. Despite having only 0.9% of the global population, we produce 15.9% of the most highly cited articles. But translation lags behind this excellent science base.

The University of Oxford took the first step towards bringing their scientists' discoveries to market by patenting them. The lead researcher, Professor Luet Wong, co-founded spinout company Oxford Biotrans to further develop this and other technologies. The company will collaborate with De Monchy Aromatics on the manufacture and marketing of this product. It is the first from Oxford Biotrans to reach a commercial scale.

If the product is successful, it will provide a return to UK plc on taxpayers' investment. The natural designation will enable the product to reach the highest price – around $4000-7000/kg, which is not far off beluga caviar ($7000-$10,000/kg). This compares to around $2000/kg for synthetic grade nootkatone while the price for valencene is around $550/kg.

17. What are you planning next?

A major advantage of the technology we have developed is that we use biological instead of chemical processes.

Similar technology could open the door to new fragrances, pharmaceuticals and natural pesticides. With this first partnership with De Monchy Aromatics, Oxford Biotrans is in a position to lead innovations in the field.

18. Would public money be better spent on technologies to help address climate change, famine or human diseases?

With greater insights into biology, it is becoming possible to replace many harsh chemicals used in various industries. Greater use of engineering in biology can help us clean up and modernise industrial processes. It can also help us find ways to produce natural compounds without using scarce resources from the environment. We’re at the start of the next industrial revolution.

Future products might include bioactive compounds like pharmaceuticals or agrochemicals.

19. Tell me more about the research funding

The research was a spinoff from Dr Luet Wong’s share in the following grant: EP/D048559/1,
http://gow.epsrc.ac.uk/NGBOViewGrant.aspx?GrantRef=EP/D048559/1

Dr Wong’s funding (around £200,000) was for fundamental studies into protein electron transfer, of which the work on p450 was part.

Dr Wong has been working in his current field for around 20 years. He studies the role of iron in biology, with a focus on the structure and function of enzymes which contain iron in the active site. He also studies the mechanisms behind the reactions that these enzymes are able to catalyse. Examples of iron-containing proteins are haemoglobin and myoglobin (essential oxygen-binding proteins in the bloodstream), cytochrome oxidase (involved in powering living cells) and cytochrome P450 (which has a key role in drug metabolism in humans).