Ornamental bulb crops as sources of medicinal and industrial natural products

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To my parents

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General Introduction

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The Netherlands is well known for its flowers, and its landscape that transforms into brightly colored blocks as the bulb flowers bloom in spring time. While tulips (*Tulipa*) are the country's most famous floral export product, a large diversity of bulb species is cultivated on large scale. These include members of the genera *Narcissus*, *Hyacinthus*, *Lilium*, *Gladiolus*, *Iris* and *Crocus*, as well as many others. Dutch ornamental bulb cultivation started in the 16th century when tulips were brought into the country from Turkey, where they had already been cultivated for a long time. The botanist Carolus Clusius (1526-1609), a Professor in botany at Leiden University played a big role in spreading and popularizing tulips in The Netherlands. Most of the other ornamental bulb crops, with the exception of *Narcissus* species, were also introduced to the Netherlands from outside Europe in the 16th century (Benschop et al., 2010). Since then intense breeding and hybridization has resulted in thousands of cultivars of each species. The Netherlands now has the highest production of ornamental geophytes in terms of hectares of land (Buschman, 2005). The demand for flower bulbs has steadily increased with the total flower bulb export for The Netherlands in 2005 reported as $756 million (Benschop et al., 2010).

Recently interest in the alternative use of agricultural crops, including ornamental bulb crops in The Netherlands has grown. A project initiated in the UK investigated the cultivation of daffodils (*Narcissus)* as a source of galanthamine (DEFRA project no. NF0612; www.nnfcc.co.uk). This caught the attention of Dutch *Narcissus* growers, who saw this as a potential novel use of one of their major existing crops. Literature studies into the chemical diversity in the Dutch ornamental bulb crops also revealed the large potential of novel uses for other ornamental crops. This was also in line with the Dutch government's plan for stimulating the development of a bio-based economy (defined as an economy in which crops and waste or leftover biomass from the agricultural sector is used for non-food industrial purposes; www.agentschapnl.nl/biobased-economy).

A project was launched to further explore the potential of Dutch ornamental crops as feedstocks or raw material for novel industrial uses. The project was supported in part by the Flowers and Food foundation (a Dutch foundation that promotes innovation in

the horticultural sector through research projects) and the Ministry of Agriculture. A major aim of the project was to set up production chains for novel industrial crops. In a follow-up project supported by the province of Zuid-Hollland, the aim was initiating a knowledge center (*kenniscentrum inhoudsstoffen*) for developing novel production chains. As a model for the practicalities of a production chain, the cultivation of *Narcissus pseudonarcissus* as raw material for the extraction of galanthamine was chosen as a case study. The aim was to use this as a model for connecting all players, from bulb to pharmaceutical formulation, and to see what knowledge is needed to establish such a system. The work presented in this thesis was done as part of these two projects, and was the results of close collaboration between academic and industry partners to coordinate all the necessary knowledge and skills.

The aim of this study was to obtain an understanding of what is needed to convert an ornamental crop to a pharmaceutical crop. This includes knowledge about regulatory issues and expectations from the industry regarding quality and safety of such crops. Based on this knowledge, a further aim was to determine how the cultivation practices of the model crop *Narcissus pseudonarcissus* needs to be adapted to meet quality requirements. This would then contribute to a validated production chain of *Narcissus pseudonarcissus* bulbs suitable for use as raw material for the extraction of galanthamine.

In this thesis, an introductory review is presented to provide background knowledge about the cultivation of plants for industrial purposes. This includes an overview of the use of plants in various industries, as well as trends in the various markets involved. Economic and regulatory issues relevant for such uses of plant material are also discussed, with a focus on the situation in the European Union. An introduction to the alkaloid galanthamine and *Narcissus* is provided, as well as an overview of its cultivation as an ornamental plant. From this overview some key points in the cultivation process are identified that may need to be adapted in the production of *Narcissus* bulbs for the pharmaceutical industry. These points are investigated in field experiments using NMR-based metabolomics as the main analytical tool. The use of this approach for plant metabolite analysis is also reviewed. Results from exploratory investigations into other ornamental bulb material for novel industrial products are presented. Finally, a general discussion and perspectives are presented at the end of the thesis.

Chapter 1. Cultivation of medicinal and aromatic plants for specialty industrial materials

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Abstract

Many specialty materials such as essential oils, pharmaceuticals, colorants, dyes, cosmetics and biocides are obtained from plants. Many species of medicinal and aromatic plants (MAPs) are cultivated for such industrial uses, but most are still wild collected. The need for renewable sources of industrial products as well as the need to protect plant biodiversity creates an opportunity for farmers to produce such crops. The production of plants as raw material for fine chemicals is different than cultivation of ornamental or food crops. This review attempts to give an overview of the use of MAPs in various industries, as well as trends in the various markets involved. Economic and regulatory issues relevant for such uses of MAP material are also discussed, with a focus on the situation in the European Union. The aim is to provide information to potential producers to help identify interesting MAPs for cultivation.

Introduction

Apart from being a source of food, many plant species are used as sources of non-food industrial products. Bulk materials such as oils, carbohydrates and fibers are obtained from various crops, and involve primary plant metabolites, which occur in relatively high levels in the plants. Such crops are typically cultivated on a large scale, and the products have a relatively low value. Another use for industrial crops is for the production of biomass as an energy source. Many plant species are cultivated for their secondary metabolites, which are used for the production of fine chemicals or specialty products. Crops in this sector are typically cultivated in smaller areas. The products are traded in lower amounts than the bulk materials, and typically have relatively higher values.

Plants used primarily for their medicinal or aromatic properties in pharmacy or perfumery are defined as medicinal and aromatic plants (MAPs) in the EU (http://www.plantaeuropa.org/pe-EPCS-hot_issues-MAP.htm). Many plants defined as MAPs are also used for cosmetic purposes, so that the definition medicinal, aromatic and cosmetic (MAC) plants would better describe such plants (Slikkerveer, 2006). In terms of production of industrial products/fine chemicals from plants, some plants defined as MAPs and MACs can also be used in the production of dyes, colorants and crop protection products. In this review the term medicinal and aromatic plants (MAPs) will be used to include all plants from which the above-mentioned fine chemicals/industrial products can be produced.

There is an increasing need for industrial products from renewable resources. Many farmers are also interested in adding value to their existing crops by utilizing agricultural waste material or by converting crops to new uses. The production of plants as raw material for fine chemicals is different than for other crops such as ornamentals, food or other industrial uses. For farmers or growers who want to produce a new crop, or use an existing crop as raw materials in a different industry, some aspects are important to consider:

- Identify the main players in the particular industry.
- Identify how trade in raw materials and ingredients is regulated and organized, nationally and internationally. The EU has many requirements, rules and regulations for the trade of MAPs that need to be followed.
- Identify future trends in the sector and where future demands might exist.
- Determine which crops can be cultivated outdoors in the particular climate and soil, and which are suitable to be grown in greenhouses.

- Calculate the costs involved in production, to determine the competitiveness of the raw materials on the market.
- Investigate the potential for the production of the final product by different means such as synthesis or by cell cultures and the influence this might have on the future price stability of the end product.
- Establish cultivation methods to ensure consistent levels of active compounds.

The Interactive European Network for Industrial Crops and their Applications (IENICA) website (www.ienica.net) describes the potential of plants as raw materials for various industrial products. While there is much potential, it mentions that the development of the non-food industrial agricultural sector is still at an early stage in Europe. For the successful development of non-food industrial crops, several issues should be kept in mind. Firstly, for non-food crops and products to be successful they should be industry/market focused. Secondly the price of the product should be competitive on the market place. The production chain should be verified, so that the industry can be assured that materials will meet their requirements and be of consistent quality. This review aims to give an overview of these points, and provides the information needed by people who want to start growing new crops of MAPs, or to use an existing crop for a new purpose. The review will focus mostly on the situation in the EU. Information was gathered from online resources such as market surveys, EU websites as well as from interviews with people working in the pharmaceutical and cosmetics industries. Where all the details cannot be provided, links or suggestions of other resources are be given where additional information can be found.

Plants as industrial crops

The following specialty materials can be obtained from plants:

- Flavors and fragrances
- Pharmaceuticals
- Herbal health products
- Dyes and colorants
- Cosmetics, personal care products
- Plant protection products
- Intermediates from which the above can be produced

Flavors and fragrances

Essential oils are highly concentrated, volatile, hydrophobic mixtures of chemicals extracted from plants. The name essential derives from the very aromatic nature of the

oils, so that the oil captures the "essence" of the plant from which it was extracted. The oils usually consist of a complex mixture of tens to hundreds of low molecular weight terpenoids. Essential oils are most commonly extracted by steam distillation, while organic solvent extraction is also sometimes used. Recently the use of supercritical carbon dioxide extraction has become increasingly popular as an alternative to organic solvent extraction.

Essential oils have characteristic flavor and fragrance properties, and many also possess other biological activities. For these reasons essential oils are used in many industries. The food industry uses them as flavorings (e.g. soft drinks, food, confectionary), the cosmetics industry uses them for their fragrance (e.g. perfumes, skin and hair care products), and the pharmaceutical industry uses them for functional properties (e.g. antimicrobial activity). Essential oils are widely used for aromatherapy and in other alternative healthcare products. About 90% of global essential oil production is consumed by the flavor and fragrance industries. This is mostly in the form of cosmetics, perfumes, soft drinks and food. The largest consumer of essential oils is the USA, followed by western European countries like France, Germany and the UK, and Japan (Holmes, 2005).

Approximately 3000 plants are used for their essential oils, with 300 of these being commonly traded on the global market (CBI, 2009a). It is difficult to obtain exact data on the global production of essential oils, but an estimate of 45 000 tonnes was reported in 2004 (CBI, 2008a). A recent estimate of the leading 20 essential oils is much higher at 104 000 tonnes (CBI, 2009b). Essential oils can be classified into three groups based on the volumes produced globally. Production of the first group exceeds 100 tonnes per year, the second group is between 50-100 tonnes, and the third group is between 1-50 tonnes (Shrinivas and Kudli, 2008). Table 1 summarizes the top 20 essential oils in each category.

Medicinal products (pharmaceuticals, herbal medicines)

Pharmaceuticals

In the conventional pharmaceutical industry, pharmaceutical companies produce medicines from compounds extracted from plant material, or use plant derived compounds as starting material to produce drugs semi-synthetically (Houghton, 2001). Examples of the former include the anti-cancer alkaloid paclitaxel from Pacific yew (*Taxus brevifolia)*, vinblastine from the Madagascar periwinkle (*Cataranthus roseus*), and digoxin from the foxglove (*Digitalis lanata*). A good example of the latter is the use of plant steroids, such as those extracted from Mexican yams (*Dioscorea* spp.), to semi-

synthetically produce steroids for use in oral contraceptives. Plant derived compounds play an important role in the production of such "single chemical entity" medicines.

Table 1. Global production of essential oils, examples of top essential oils in each production category (Compiled from Table in (CBI, 2009a), and Shrinivas & Kudli 2009).

Production above 100 tonnes	**Production 50-100 tonnes**	**Production 1-50 tonnes**
Orange (*Citrus sinensis, C.aurantium*)	Spike lavender *(Lavandula latifolia)*	Caraway *(Carum carvi)*
Cornmint (*Mentha arvensis*)	Sage *(Salvia sclarea, S.officinalis, S.lavandulifolia)*	Cumin *(Cuminum cyminum)*
Lemon (*Citrus limon*)	Pimenta *(Pimenta dioica)*	Violet leaf *(Viola odorata)*
Eucalyptus *(Eucalyptus globulus)*	Thyme *(Thymus zygis, T.vulgaris)*	Black current *(Ribes nigrum)*
Peppermint *(Mentha piperita)*	Marjoram *(Origanum majorana)*	Neroli *(Citrus bigaradia)*
Citronella *(Cymbopogon winterianus)*	Basil (*Ocimum basilicum)*	Rose *(Rosa damscena, R.centifolia)*
Clove leaf *(Syzygium aromaticum)*	Juniper berry (*Juniperus communis*)	Carrot seed *(Daucus carota)*
Chinese sassafras *(Cinnamomum micranthum)*	Celery (*Apium graveolens)*	Angelica root, seed, herb *(Angelica archangelica)*
Lime *(Citrus aurantifolia)*	Palmarosa *(Cymbopogon martinii)*	Buchu *(Barosma betulina)*
Lavandin (*Lavandula hybrida)*	Rosewood *(Aniba rosaeodora)*	Jasmine *(Jasminum grandiflorum)*
Patchouli *(Pogostemon cablin)*	Sandalwood (*Santalum album)*	Tuberose *(Polianthes tuberosa)*
Scotch spearmint *(Mentha spicata)*	Guaiac wood *(Bulnesia sarmientoi)*	Tarragon *(Artemisia dracunculus)*
Eucalyptus *(Eucalyptus.citriodora)*	Ylang-ylang *(Cananga odorata)*	Galbanum *(Ferula gummosa)*
Chinese cedarwood *(Chamaecyparis funebris)*	Chamomile, Roman and blue *(Anthemis nobilis, Chamomilla recutita)*	Bay leaf *(Pimenta racemosa)*
Litsea cubeba *(Litsea cubeba)*	Srilanka cinnamon *(Cinnamomum zeylanicum)*	Hyssop *(Hyssopus officinalis)*
Native spearmint *(Mentha gracilis)*	Cardamom *(Elettaria cardamomum)*	Muhuhu *(Brachylaena hutchinsii)*
Texas cedarwood *(Juniperus virginiana)*	Gurjun balsam *(Dipterocarpus spp.)*	Calamus *(Acorus calamus)*
Star anise *(Illicium verum)*	Bay laurel leaf *(Laurus nobilis)*	Carnation (*Dianthus caryophyllus)*
Mandarin *(Citrus reticulata)*	Labdanum leaf *(Cistus ladanifer)*	Mimosa *(Acasia dealbata, A.mearnsii)*
Vetiver *(Vetiveria zizanoides)*	Cajeput *(Melaleuca Leucadendron)*	

Table 2. Examples of commonly used isolated compounds from plants.

Compound	Chemical class	Source	Therapeutic use
Morphine, codeine	Alkaloids	*Papaver somniferum*	Analgesic, antitussive
Digoxin	Steroidal glycosides	*Digitalis lanata*	Heart disorders
Atropine	Tropane alkaloids	*Atropa belladonna*, *Hyoscyamus* spp.	Antispasmodic, pupil dilator
Scopolamine	Tropane alkaloids	*Atropa belladonna*, *Hyoscyamus* spp.	Sedative, against motion sickness
Paclitaxel	Diterpene alkaloid	*Taxus* spp.	Anticancer (ovarian and others)
Quinine	Quinoline alkaloid	*Cinchona* spp.	Antimalaria
Vinblastine, vincristine	Bis-indole alkaloid	*Catharanthus roseus*	Anticancer (leukemia)
Camptothecin	Indole alkaloid	*Camptotheca acuminata*	Antineoplastic
Galanthamine	Isoquinoline alkaloid	Various members of *Amaryllidaceae*	Against mild Alzheimer's disease, Dementia
Physostigmine	Carbamate alkaloid	*Physostigma venenosum*	Alzheimer's disease, glaucoma
Artemisinin	Sesquiterpene lactone	*Artemisia annua*	Antimalaria
Podophyllotoxin	Lignan	*Podophyllum* spp.	Antiviral, antineoplastic
Pilocarpine	Imidazole alkaloid	*Pilocarpus* spp.	Glaucoma, xerostomia

More than 25% of the pharmaceutical drugs used in the world today are derived from plant natural products (Farnsworth, 1979; Schmidt et al., 2008). Table 2 lists some more examples of plant-derived drugs.

Herbal medicines

Medicines in the form of extracts, teas, tinctures or capsules produced from MAPs are referred to as phytopharmaceuticals, phytomedicines or herbal medicines in Europe, and botanical drugs in the United States. In many European countries there is not much difference between pharmaceutical and phytopharmaceutical companies. Medicines are

produced to high standards and must undergo clinical evaluation of safety and efficacy (Schmidt et al., 2008). Clinical trials have been performed for many herbal medicines and for the most part it is known which constituents are responsible for the medicinal effect (Houghton, 2001). Phytopharmaceutical medicines are standardized in terms of the active constituents. Examples of popular phytopharmaceuticals are *Gingko biloba* extract to improve cognitive function (O'Hara et al., 1998), St. John's Wort for treatment of mild depression (Bilia et al., 2002), ginseng as general tonic and cognitive enhancer (Attele et al., 1999), ginger against nausea and vomiting (Ernst and Pittler, 2000) and saw palmetto for the treatment of symptomatic benign prostatic hyperplasia (Kaplan, 2005). Some more examples of popular herbal medicines are shown in Table 3.

Nutraceuticals/Functional foods

Recently a group of products called health products, nutraceuticals or dietary supplements have gained popularity. There are no universally accepted definitions for functional food and nutraceuticals, but generally they are described as foods (fortified with added ingredients or not) with health benefits beyond basic nutrition (Wildman and Kelley, 2007). Sometimes the plant materials used to prepare health products are similar to those in the phytopharmaceutical industry, but products are marketed as nutraceuticals to avoid the costly and time-consuming process of licensing a medicinal product (Kuipers, 1997). Often limited health claims are made about a food component when clear evidence for its role in reduced risk of a disease or health benefit is emerging, but not strong enough to meet standards set by regulatory authorities such as the FDA (Wildman and Kelley, 2007). Garlic and ginseng extracts for example have been developed as nutraceuticals. Other examples include the use of red grape constituents for its antioxidant properties, lycopene from tomatoes, and broccoli for its cancer preventative properties. Even though the relatively recent trend has opened up opportunities for value addition and new product development in the food and pharmaceutical industries (Childs, 2009), the crops involved are mostly food plants and will not be covered further in this review, which aims to address more non-food crops.

Dyes and colorants

Compounds extracted from plants can be used as natural colorants or dyes. Plants were traditionally sources of natural dyes used to color textiles. This was replaced by synthetically produced dyes in the 19th century. Recently however, an increasing public awareness of the detrimental environmental impact of synthetic dye production, together with a need for sustainable sources of dyes has led to natural products becoming popular again. Since the 1990s much research into the re-introduction of

natural dyes has been initiated (Bechtold et al., 2003; Gilbert and Cooke, 2001). The production of natural dyes from waste materials of the timber, food and other agricultural industries is of particular interest (Bechtold et al., 2007a; Bechtold et al., 2007b). According to a recent report, imports of natural dyes into the EU has increased by 3% from 2002 to 2007, while imports of synthetic dyes decreased by 2% per year in that time (CBI, 2009c). Natural dyes are used to color natural fibers such as wool, cotton, linen and hemp. They are also increasingly used in other industries, for example in paints and varnishes, cosmetics, food, in the eco-building industry, and in painting restoration (CBI, 2009c). Natural dyes of various chemical classes can be obtained from organs of specific plants. Table 4 shows a list of some examples.

Cosmetics

A cosmetic product is defined as "a substance or preparation intended for application to any external surface of the human body, teeth or mouth for the purpose of cleaning, perfuming or protecting them, keeping them in good condition or changing their appearance" (Dweck, 1996). Cosmetics companies produce different kinds of products; beauty and personal care, hair care, perfume and fragrances, and the recently growing group of cosmaceuticals (products that contain one or more bioactive compound and are intended to enhance health and beauty) (CBI, 2008b). Some important plant-derived ingredients used in the manufacture of cosmetics are oils, fats and waxes, essential oils and oleoresins, plant extracts and colorants. Vegetable fats and oils are composed of triglycerides, and are usually extracted from the seeds of oilseed plants. These substances are referred to as oils or fats depending on whether they are liquid or solid at room temperature. Solid fats with a brittle texture are also referred to as waxes (CBI, 2008a). These ingredients have numerous roles in the final cosmetic products, such as a fragrance or coloring function, moisturizers, thickening agents and stabilizers. Many plant extracts are used in cosmetics for their functional properties, such as free-radical scavenging, sun protection, whiteners and anti-microbial effects (Aburjai and Natsheh, 2003). Essential oils are discussed in more detail in section 2.1, but examples of these commonly used in cosmetics together with other popular plant-derived cosmetics ingredients are shown in Table 5.

Table 3. Examples of commonly used herbal medicines from plants.

Plant	**Active compounds**	**Source**	**Therapeutic use**
Echinacea	Alkylamides and other compounds	*Echinacea purpurea*	Immune modulator
Ginseng	Ginsenosides, eleutherosides	*Panax ginseng*	Fatigue, stress
Saw palmetto	Phytosterols, fatty acids	*Serenoa repens*	Benign prostatic hyperplasia
Ginkgo	Ginkgolides (terpene trilactones), flavonol glycosides	*Ginkgo biloba*	Mental fatigue, cognitive decline
St. John's wort	Hyperforins	*Hypericum perforatum*	Mild depression
Valerian	Iridoid glycosides, terpenoids, valerianic and isovaleric acid	*Valeriana officinalis*	Anxiolytic, sleep improvement
Garlic	Allicin	*Allium sativum*	High cholesterol, hypertension, respiratory infections
Feverfew	Sequiterpene lactones	*Tanacetum parthenium*	Migraines, inflammation
Black cohosh	Triterpene glycosides	*Cimicifuga racemosa*	Premenstrual symptoms, dysmenorrhea

Table 4. Plants containing natural dye/colorant compounds used currently or in the past.

Common name	**Source plant**	**Dye/colorant chemical class**	**Color**
Marigold	*Tagetes patula*	flavonoids	yellow
Walnut	*Juglans regia*	naphthoquinone	brown
Henna	*Lawsonia inermis*	naphthoquinone	red
Woad	*Isatis tinctoria*	alkaloid	blue (indigo)
Dyer's knotweed	*Polygonum tinctorium*	alkaloid	blue (indigo)
Madder	*Rubia tinctorum*	anthraquinone	red-brown
Barberry	*Berberis vulgaris*	alkaloid	yellow-brown
Goldenrod	*Solidago* spp.	flavonoid	yellow-olive
Hollyhock	*Alcea rosea*	anthocyanin	brown-green
Privet	*Ligustrum vulgare*	anthocyanin	blue-green
Ash tree	*Fraxinus excelsior*	flavonoid	beige-black
Sticky alder tree	*Betula alnus*	tannin	beige-black
Turmeric	*Curcuma longa*	polyphenol	bright yellow
Annatto	*Bixa orellana*	carotenoids	yellow-orange
Oil palm	*Elaeis guineensis*	carotenoids	golden yellow-orange
Safflower	*Carthamus tinctorius*	flavonoid	yellow-red
Paprika	*Capsicum annuum*	carotenoids	orange-red
Red beet	*Beta vulgaris*	betanine	pink-red
Grapes	*Vitis vinifera*	anthocyanin	red-blue
Spinach	*Spinacia oleracea*	chlorophyll	green

Plant protection products

There is a long history of use of plants as sources of plant protection products against insects, microbial pathogens and weeds. Records of plant-derived insecticides date back more than 2000 years ago in China, India, Egypt and Greece (Isman, 2006). In the middle of the 20th century synthetic chemical pesticides became dominant and the use of botanicals dwindled. The potent and fast action of the synthetics led to great increases in yields of crops in many parts of the world. However with these improvements came unwanted effects such as destruction of non-target organisms, disruption of pollination, groundwater contamination, chronic and acute human poisoning, and resistance in pest populations. In recent years concerns over these problems have resulted in the banning or restriction of dangerous compounds, and a move towards the use of less harmful ones. Plants are an attractive source since their metabolism has adapted to deal with pests in their environment. Some compounds or extracts can be used directly, and others serve as precursors for the production of agents used as weed, insect or plant pathogen protectants (Copping and Duke, 2007; Dayan et al., 2009).

Various products derived from plants are currently being used to control insect pests, microbial pathogens and weeds. The best known ones are the pyrethrins extracted from *Tanacetum cinerariaefolium* achenes, rotenone isolated from *Derris lanchocarpus* roots and azadirachtins isolated from seeds of the Indian neem tree (*Azadirachta indica)* (Guleria and Tiku, 2009). The essential oils of some well-known aromatic plants have also been applied to plant protection, and can be found in commercial preparations (Isman, 2006). Table 6 summarizes various plant-derived products that are currently being used and are available on the market in at least some countries.

Table 5. Examples of plant-derived ingredients commonly used in cosmetic products.

Ingredient type	Ingredient	Source	Function
Oils	Castor oil	*Ricinus communis*	Moisturizers,
	Jojoba oil	*Simmondsia chinensis*	emollients
	Almond oil	*Prunus dulcis*	
	Sesame oil	*Sesamum indicum*	
	Avocado oil	*Persea Americana*	
	Apricot kernel oil	*Prunus armeniaca*	
	Rapeseed oil	*Brassica napus*	
	Linseed oil	*Linum usitatissimum*	
	Sunflower seed oil	*Helianthus annuus*	
	Palm oil	*Elaeis guineensis*	
	Coconut oil	*Cocos nucifera*	
Fats and waxes	Cocoa butter	*Theobroma cacao*	Moisturizer,
	Carnauba wax	*Copernicia prunifera*	emulsifier
	Candelilla wax	*Euphorbia* spp.	
Gums	Gum Arabic	*Acacia* spp.	Stabilizer, adhesive
	Gum tragacanth	*Astralagus* spp.	agent, jelly lubricant,
	Guar gum	*Cyamopsis* spp.	suspending agent,
	Locust bean gum	*Ceratonia siliqua*	thickeners, binders
Essential oils	Patchouli oil	*Pogostemon cablin*	Fragrance
	Citronella oil	*Cymbopogon*	
	Sandalwood oil	*winterianus*	
	Bergamot oil	*Santalum album*	
	Rosemary oil	*Citrus aurantium*	
	Rose oil	*Rosmarinus officinalis*	
	Mint oil	*Rosa damascena*	
	Jasmine oil	*Mentha piperita*	
	Vetiver oil	*Jasminum officinale*	
		Chrysopogon	
		zizanioides	
Extracts and saps	Aloe sap	*Aloe vera*	Moisturizer,
	Acai fruit extract	*Euterpe oleracea*	emmolients
	Baobab fruit extract	*Adansonia* spp.	
	Guarana extract	*Paullinia cupana*	
Colourants	Indigo extract	*Indigofera* spp.	Colouring
	Curcuma extract	*Curcuma* spp.	
	Henna extract	*Lawsonia inermis*	
	Marigold extract	*Tagetes* spp.	

Sources of plant material for the industry

Wild collection versus cultivation

Plants used as raw materials for industry are supplied in two ways, through collection in the wild and via cultivation. Each of these methods of supplying plant material has advantages and disadvantages. For the pharmaceutical and other medicinal industries, cultivated plant material is preferred as it is easier to control the whole supply chain and chemical variation will be less. With the use of cultivated plants, problems such as adulteration or misidentification of material, is mostly eliminated. It is also easier to adhere to quality standards and have less batch-to-batch variation as the plants are grown under controlled conditions. A more reliable supply of raw material is also probable and the price of the material will be more stable than with wild collected material purchased on the open market. The disadvantages of cultivation are that the raw material is usually more expensive than wild harvested plants, since investment is required before production can start and during the cultivation process. Also, many plant species are not easy to cultivate, or they are slow growing and thus not economically feasible for most growers (CBI, 2008b; Schippmann et al., 2006).

Since wild collected material needs no infrastructure and investment, it is usually less expensive than cultivated materials. When the market size is relatively small for a specific raw material it would also not be economically feasible to cultivate the plant. There is also a perception in many traditional medicinal systems that wild grown plants are more potent or effective than cultivated plants. The main problems with wild harvested plants are that there is a risk of adulteration with plants from the same genus leading to the reduction or loss of the active components in the material. Plants with similar morphological characteristics growing in the same region might also be collected leading to the common problem of misidentification. Contamination may occur through non-hygienic conditions during or after harvesting (CBI, 2008b; Schippmann et al., 2006). Sustainability can be a problem with wild harvesting, and wild populations may be depleted. Plant species that have large habitats are fast-growing and whose foliage alone is harvested are usually not a problem. However, species that are slow-growing, have a limited distribution or whose roots or bark are harvested are susceptible to over-harvesting. While wild-harvested plants are perceived to be less expensive because of the lack of overhead investment, it should be remembered that neither the cost of replacing the wild stocks/resources, nor the extra costs of quality control are included (Cunningham, 1997).

Table 6. Natural compounds or extracts that have been well characterized and/or have found commercial application as plant protection products.

Type	Product	Source	Action
Pure compound	Cinnamaldehyde	*Cassia tora*	Preventative treatment against various fungi
	Nicotine	*Nicotiana* spp.	Insecticide against wide range of insects
	Azadirachtin/dihydroazadirachtin	*Azadirachta indica*	Deters many insect genera, effective against certain fungi.
	Eugenol	*Eugenia caryophyllus*, *Laurus* spp.	Deters wide range of insects, herbicidal at high doses.
	Karanjin	*Derris indica*	Control of mites, chewing and sucking insects, some fungi.
	Rotenone	*Derris lanchocarpus* and *Tephrosia* spp.	Selective non-systemic insecticide. Traditionally used as fish poison.
Plant-derived oil	Jojoba oil	*Simmondsia californica*	Against whitefly, powdery mildew
	Clove essential oil	*Eugenia caryophyllus*	Non-selective foliar contact herbicide.
	Pine oil	*Pinus* spp	Non-selective contact herbicide
	Oil of anise	*Pimpinella anisum*	Irritant to insects, alters leaf surface, can cause suffocation.
	Soybean oil	*Glycine max*	Irritant to insects, alters leaf surface, can cause suffocation.
	Eucalyptus oil	*Eucalyptus* spp.	Irritant to insects, alters leaf surface, can cause suffocation.
	Mixtures of thyme, wintergreen, marigold oils	*Thymus vulgaris*, *Tagetes*, *Gaultheria* spp.	Irritant to insects alters leaf surface, can cause suffocation.
Plant-derived acids	Pelargonic acid	Geraniaceae family	Broad spectrum contact herbicide against weeds, mosses, liverworts.
	Fatty acids	Blends of fatty acid salts from various plants	Control of moss in greenhouses, on roofs, fences.
	2-phenethyl propionate	*Mentha* spp.	Herbicide, some antifungal activity insecticide

	Citric acid	Various plants	Against wide range of insects.
	Formic acid	Various plants	Kills mites directly
Extracts	Milsana	*Reynoutria sachalinensis*	Stimulates plants natural defense mechanism, against fungal and some bacterial pathogens.
	Pink plume poppy extract	*Macleaya cordata* (contains alkaloids sanguinarine, protopine and others)	Against foliar fungal pathogens, mode of action not clear.
	Pyrethrum (oleoresin)	*Tanacetum cinerariifolium* and *Pyrethrum cinariifolium*. (Contains pyrethrins, cinerins and jasmolins)	Contact action against wide range of insects. Often used in combination with other products.
	Ryania extract	*Ryania speciosa* (contains alkaloids)	Against codling moth, corn borer, citrus thrips. Used to treat infected crops, quick effect makes it useful to stop boring insects.
	Sabadilla	*Schoenocaulon officinale*. (Contains alkaloids cevadine, veratridine, esters of alkamine and veracine)	Contact action against thrips causing paralysis and death.
	Capsicum oleoresin	*Capsicum frutescens*	Insect and mite repellant, claimed to control soil fungi, insects, mollusks, nematodes. Also repels animals due to irritant effect.

For successful large-scale cultivation of MAPs, high quality raw material should be produced using low input cultivation methods so that it can compete in the international market and with plants collected in the wild. Alternatively where much investment is needed to set up cultivation, plant material with a high value should be grown. While it is possible to obtain information on the volumes of plant material exported from a country, it is not always clear how much of this is cultivated or wild harvested. Within Europe, an area of more than 100 000 hectares is under cultivation of plants used in the pharmaceutical or cosmetic industries (EHGA Europam 2006, www.europam.net/index.php?option=com_content&view=article&id=6&Itemid=11). This number is based on incomplete data and the area is likely to be larger. France, Germany, Austria and Finland are the largest producers in Europe with the largest area under cultivation. Eastern European countries are also important as lower labor costs make cultivation less expensive, so that their raw materials are more economically competitive.

Sources of plant material for different industries

Sources of raw plant material for essential oil production

European countries were traditionally the production area for essential oils, due to long traditions of perfume making. At present the US and developing countries are dominant in the production of the most important essential oils. EU countries like France and Italy play an important role for high-yielding oils derived from plants whose cultivation, harvesting and processing can be fully mechanized, making production competitive with countries where labor costs are low (CBI, 2009b). The major essential oil producing countries are (in descending order) USA, China, Brazil, Turkey, Indonesia, Morocco, Hungary, Bulgaria, India, France, Italy, Spain and Egypt (CBI, 2009a). The USA accounts for 24% of the production, China for 20% and Brazil for 8%. The remaining countries all produce 5% or less of the world's essential oils in terms of volume.

Sources of raw plant material for medicinal product production

In general the medicinal and cosmetic industries in European countries still rely mostly on wild-collection as sources of raw MAP material (Vines, 2004). The high costs of domestication and cultivation are the main reasons for this, together with the fact that cultivation of some species is not possible. The exception is large pharmaceutical companies, who need to produce drugs on a large scale and have to comply with very strict quality control standards throughout the production process. Cultivation for large companies is usually done under contract (CBI, 2008b). This ensures a reliable supply

and volume of material, as well as easier standardization of active ingredients. A good example is the cultivation of *Papaver somniferum* in Tasmania for the production of opium alkaloids for the pharmaceutical industry. Two large processing companies contract hundreds of farmers to cultivate poppies on a large scale (Fist, 2003; Hagel et al., 2007). While the scale on which plants are cultivated for such pharmaceutical companies is large, only a small proportion of the thousands of medicinal plant species are cultivated in this way. Medicinal plants are also cultivated for the production of phytopharmaceuticals in countries like Germany, Switzerland and Austria where herbal medicines are well defined, production is regulated and quality is well-controlled. Cultivation is usually contracted to farmers in a region where the climate is favorable, which may or may not be close to the extraction/processing facilities. European companies contract farmers to grow plants in places like India, South Korea, and more recently eastern European countries like Poland where labor costs are relatively low.

Sources of raw plant material for dye and colorant production

A challenge in the production of natural dyes is supplying large enough quantities needed for mass production of products (CBI, 2009c). At present the main exporting countries of natural dyes are China, Peru and India. In the EU there is only limited plant cultivation for dye raw material due to high labor costs and climatic conditions. The cultivation of plants for food colorants is more widespread in the US and Europe. Crops grown on large scale for food are also used to extract natural colorants, such as grapes in Italy and paprika in Spain and Hungary (CBI, 2009c). Raw materials for food colorants not available in the EU include turmeric, marigold, palm oil and safflower.

Sources of raw plant material for cosmetic production

Sources of raw plant material for cosmetics are mostly similar to those of medicinal products. When a steady supply of material is needed in large amounts, companies set up cultivation schemes under contract. Much of the raw material used is still harvested in the wild, however. Sources of essential oils and coloring products are described in sections 3.2.1 and 3.2.3, and are the same in the cosmetics industry. Plant-derived gums are obtained from African countries like Sudan, Chad and Nigeria, but fluctuating supply and quality make cosmetics producers often use other gum types. The exception is locust bean gum that is produced in Mediterranean countries such as Greece, Spain and Italy. Fats and waxes (e.g. coconut oil, cocoa butter) are mostly imported from Ivory Coast, Ghana, China and Indonesia. The specialty waxes carnauba wax and candelilla wax come from Brazil and Mexico, respectively (CBI, 2008a).

Sources of raw plant material for biocide production

Raw material for the production of plant-derived pesticides can be obtained through wild-collection of plants or cultivation. Cultivation is the most suitable option as a large continuous supply of raw material is needed for commercial production. At the moment plants available on agricultural scale for biopesticide production include neem, pyrethrum and many plants with essential oils.

Plant cell and tissue culture as sources of industrial products

An alternative way to obtain plant metabolites on large scale is to use biotechnological methods (Verpoorte *et al.* 2002). Plant cells can be cultured as a suspension in bioreactors, or differentiated cells can be grown as tissue or organ cultures for the production of secondary plant metabolites (Srivastava and Srivastava 2007). Not all plant cells produce secondary metabolites in such cultures, but with optimization of growth conditions and with proper elicitation, many plants cell cultures can produce these metabolites at levels comparable to or higher than in the plant (Georgiev *et al.* 2009). The use of such methods has been shown to be technically possible, but economic feasibility can be limited for industrial products unless they are of very high value. Some examples of secondary plant metabolites being commercially produced for pharmaceutical use in cell culture are paclitaxel (*Taxus* spp.), ginseng saponins (*Panax ginseng*), scopolamine (*Duboisia* spp.), protoberberines (*Coptis japonica, Thalictrum minus*), rosmarinic acid (*Coleus blumei*) and geraniol (*Geramineae* spp.) (Kolewe *et al.* 2008). Biotechnology and plant cell culture is not yet as widely applied in cosmetics as in pharmaceuticals. Some of the rare examples of plant metabolites produced in cell culture for cosmetic use include the whitening agent arbutin (*Catharanthus roseus*) and the cosmetic pigments carthamin (*Carthamus tinctorius*) and shikonine (*Lithospermum erythrorhizon*) (Schürch *et al.* 2008). A system for apple (*Malus domesticus*) cell suspension culture was developed for the production of extracts to be used in cosmetic products (Schürch *et al.* 2008). A recent trend is for cosmetic products containing plant "stem cells" that have rejuvenating or invigorating effects on the human skin. These so-called stem cells refer to plant cells produced in suspension or callus culture added to the product. For biocides, hairy root culture has been shown to be promising for the production of azadirachtin, phytoecdysteroids and nicotine (George et al., 2000). However, scaling up of the process for commercial production of pure compounds is not yet economically feasible.Research efforts continue to improve the understanding of plant metabolic pathways and their regulation (Verpoorte et al. 1999, Roberts 2007). This knowledge should allow for higher yields and increased efficiency of cell and tissue culture processes, making them promising methods for the sustainable production of industrial plant-derived metabolites.

Organization of trade in MAP material

Trade in medicinal plants takes place on three main levels. There is trade at a national level within countries, trade across national borders within a continent, and the formal export trade (Cunningham, 1997). Of the thousands of medicinal plant species in the world, relatively few are traded in the formal export sector but volumes may be large for some. As this is the sector most relevant for industry using medicinal plants as raw material in Europe, the structure and organization of this form of trade will be focused on.

Most plant material (cultivated or wild collected) is traded in dried form, except in some cases where the desired compounds are sensitive to the drying process (e.g. terpenoids). In the country where the plant material originates, local dealers source material from local collectors and growers. From the point of harvest or collection, plant material is purchased and can pass via a number of regional traders, local dealers or village cooperatives to larger plant trading and exporting companies. Sometimes special collectors or agents sourcing rare or specialty plants buy plants directly from collectors for export (Hamilton, 1992).

Raw material may be exported for processing in other countries or processing may take place in the source country. In the past developing countries almost exclusively exported raw material to European countries but several countries show an increase in processing activities before exporting material (De Silva, 1997).

Large trading companies import plant material. These companies have large warehouses where huge stocks of material are kept. The most important places for importing medicinal plant material are Hong Kong, Tokyo and New York, and Hamburg for Europe (Kuipers, 1997). This is also where the major trading companies are located. Exporters, importers and wholesalers may specialize in a few kinds of raw material, or deal with hundreds of plant species (CBI, 2008b). The large trading companies have a central role in the medicinal plant trade. They significantly influence the pricing of material due to the large scale on which they purchase it (Kuipers, 1997). They also usually have the facilities to perform quality control tests required by industries that use the material. This gives them a further position of power as smaller companies are not able to afford quality control tests and thus rely on them for this service.

Wholesalers may perform some processing steps. Often, however, plant material is sold to processing companies where material is processed before being passed to the end-product manufacturers. Material already processed in the source country may be further

processed in Europe. Processing steps may also be carried out by the end-product manufacturer. The processing industry consists of several parts, each performing specific tasks. These include extraction, production of nut and seed oils, processing of fresh materials (drying, sieving, distillation, juicing, density adjustment, extraction), and wholesale distributors with particular value-added capabilities (e.g. performing quality control tests such as pesticide residue detection, mycotoxin trace detection).

Traceability is very important to the manufacturers of medicinal and cosmetics products. In the EU traceability and strict documentation requirements such as Good Agricultural Practice (GAP) and Good Manufacturing Practice (GMP) are becoming more important. Traceability is made difficult when raw materials are sourced from many different places and has passed through many hands in the process. For this reason, more direct sourcing of materials is desired.

It is not easy to obtain accurate information about the volumes of MAPs involved in international trade. This is in part due to the fact that the plant species involved are often used in more than one industry, so a particular volume of raw material produced may end up being used for a phytopharmaceutical, cosmetic or food product. Also many of the companies involved in the trade of MAPs are not very open to share information about specific numbers (Holmes, 2005; Lange, 2006). One way to obtain information, however limited, on some MAP trade Figures is from the COMTRADE database compiled by the UN Statistics Division (http://comtrade.un.org). This database contains foreign trade Figures of about 180 countries since 1962. In the database goods are classified according to various classification systems. For MAPs the two most important ones are the Standard International Trade Classification (SITC) and the Harmonized Commodity Description and Coding (HS) systems. The SITC was developed by the United Nations Statistical Division (New York). It has been revised numerous times and while the latest one is from 2006 (SITC4, 2006) the previous system is also still used (SITC3, 1986). Most MAPs are classified in section 2 of this system (Crude materials, inedible, except fuels) while a few are in section 0 (Food and live animals). Within section 2 some high value products such as licorice roots and ginseng roots are placed in their own division, while the rest are classified as "other". A new economic classification was developed and applied from 1988. The Harmonized Commodity Description and Coding system (HS), developed by the World Customs Organization (WCO) was aimed at improving the recording and analysis of statistical data on an international level, as well as making trade statistics more comparable between countries. The European Union has adopted a classification system based on the HS system, which is called the Combined Nomenclature (CN) system. It is basically the same as the HS, except that some subdivisions are specific to requirements of external trade statistics, agricultural laws and customs tariffs in the EU (Lange, 2006).

The HS system is more detailed than the SITC system. In the previous revision of the SITC.3 system licorice roots were given a special subdivision, which has been removed in the latest version (SITC4, 2006). The HS system also changes details of subdivisions regularly. Different countries often use different classification systems, including older versions of the ones described (Lange, 2006). All of this creates a situation where data are often not comparable, which makes it very challenging to portray an accurate overview of trade in raw MAP materials (Lange, 2004). In spite of the shortcomings and difficulties in obtaining some trade information, the COMTRADE database can still provide a general overview of what is going on in the world. Lange (2006) provided a summary of the twelve leading countries of import and export of MAP material (classified as "pharmaceutical plants" (SITC.3: 292.4 = HS 1211)) from the period 1991 to 2003. To compare the situation over the last few years, the same data was obtained from the database for the years 2004-2008, and is presented in Table 7.

In terms of quantity of MAP material imported, Hong Kong (as a Special Administrative Region of China) and the USA are still the most important countries in the world. Even though Hong Kong is still ranked second for the time period from 2004 to 2008, the average amount of imported material has decreased since the period 1991 to 2003. The average amounts imported have also decreased for Japan and France. All the other average import quantities have increased over the last few years. In particular the amount imported into the UK has increased quite dramatically. China remains the country with the highest average quantity of exported MAP. The average export amounts have increased for most countries and remained about the same for the USA. Albania and Bulgaria showed a slight decrease in average exports, while Hong Kong's exports decreased substantially. Poland was not included in the list of top 12 exporting countries in the previous time period, but is ranked sixth for recent years.

Germany is a very important hub of trade in MAPs for the EU and the world. Material is imported from all over the world, and exported to many (147) countries (Lange, 2006). Almost half of the material imported into Germany is from other European countries, the main ones being Bulgaria, Poland and Hungary. Other important source countries are India, Sudan, Chile and Egypt. The majority of exports go to a few countries such as Austria, Switzerland and the USA. Mostly raw material is imported into Germany, where the degree of processing is increased before export. This is reflected in the higher value of materials exported from Germany. The port of Hamburg is the most important for trade in Germa

Table 7. The top twelve MAP importing and exporting countries (SITC.3: 292.4 = commodity group HS 1211), listed according to descending order of average trade volumes between 2004 and 2008. Source: COMTRADE database, United Nations Statistic Division, New York.). Data for 1991-2003 from Lange (2006).

1991-2003 Imports			2004-2008 Imports			1991-2003 Exports			2004-2008 Exports		
Country	**Tonnes**	**Value (US$)[a]**	**Country**	**Tonnes**	**Value (US$)[a]**	**Country**	**Tonnes**	**Value (US$)[a]**	**Country**	**Tonnes**	**Value (US$)[a]**
Hong Kong	59 950	263 484	USA	62 038	220 638	China	150 600	226 038	China	204 082	348 821
USA	51 200	139 379	Hong Kong	48 168	167 298	Hong Kong	55 000	201 021	India	47 551	93 450
Japan	46 450	131 031	Germany	47 814	139 684	India	40 400	61 665	Mexico	38 262	38 081
Germany	44 750	104 457	South Korea	34 732	47 563	Mexico	37 600	14 257	Egypt	24 600	34 151
South Korea	33 500	49 889	Japan	27 937	118 514	Germany	15 100	68 243	Germany	18 076	99 349
France	21 800	51 975	China	27 754	34 808	USA	13 050	104 572	Poland*	15 775	47 183
China	15 550	41 602	France	20 336	72 471	Egypt	11 800	13 476	Hong Kong	15 011	81 698
Italy	11 950	43 006	UK	17 895	51 006	Bulgaria	10 300	14 355	USA	13 919	95 704
Pakistan	10 650	9 813	Spain*	15 670	44 337	Chile	9 850	26 352	Chile*	11 627	27 656
Spain	9 850	27 648	Italy	13 436	59 582	Morocco	8 500	13 685	Morocco*	10 796	19 463
UK	7 950	29 551	Malaysia	11 348	44 058	Albania	8 050	11 693	Bulgaria	9 338	21 088
Malaysia	7 050	38 685	Pakistan	11 045	4 733	Singapore	7 950	52 620	Albania	7 770	17 084

*: average from 2004-2007; data from 2008 not available. [a]in thousands of US dollars

The USA is an important trade center for the Americas. As for Germany, imports consist mainly of raw materials and higher export values indicate a high degree of processing before export. Imports are mainly from India, China, Mexico and Egypt, while materials are exported mainly to North and South American countries. Hong Kong used to be the most important center for trade in MAPs in east and Southeast Asian countries. Since it has become a part of China as a special administrative region (SAR), it has become less important. Trade in China has increased steadily and China now outranks Hong Kong in terms of volumes of material exported. Hong Kong remains an important place of distribution of MAP materials to other countries in the region, but China has now become the main hub of trade in MAPs in Asia.

Lange (2006) describes countries as either consumers or sources of MAPs depending on their net imports. Consumer countries import mainly raw materials, which are further processed in the country's industry, and then sold on the local market or exported as finished products. These countries have a high net import, and examples include Japan, the USA, Republic of Korea and Germany. Source countries have high negative net imports, and are the important providers of material in the world. The most important source country is China, and others include India, Bulgaria, Mexico, Chile, Egypt and Morocco.

The trade statistics obtained from the COMTRADE database gives an overview, but lacks detailed information as they cover very different product groups. The Centre for the Promotion of Imports from Developing Countries (CBI) Market Survey (CBI, 2008b) provides some more detailed information about the import of MAP and related materials into the EU. In this report, three groups are distinguished; the MAP raw material, vegetable saps and extracts, and vegetable alkaloids (purified chemicals such as opium alkaloids, rye ergot alkaloids and *Cinchona* alkaloids). In 2007 just over 40% of raw materials imported into EU countries originated in other EU countries. Germany and Poland were the main suppliers. Developed countries outside the EU (e.g. USA and Israel) accounted for 16% of the total imports, while the remaining 41% was from developing countries. Of these, China and India produced most materials. The main source of vegetable saps and extracts imported into the EU was other EU countries (42%). In 2007 Spain was the leading source, followed by France. The USA again was the main developed country source outside the EU, with Turkey being the main developing country source for this category. Total imports of saps and extracts from developing countries were 31% in 2007. For the category vegetable alkaloids, the main source was from countries inside the EU (59%). Germany, Austria and the UK were the leading suppliers in 2007. For outside EU developed countries, Switzerland was the main supplier followed by the USA. In this category, only 7% of alkaloid imports were from developing countries such as India and China.

In the past raw materials were sourced from all over the world, while the processing of these materials occurred in European countries or the USA. Now a growing trend is the processing of raw materials in the source countries before export to western countries. While the extraction and processing in western European countries are still dominant, a small but growing number of suppliers in countries like China, India, Mexico and Malaysia are offering processed ingredients (CBI, 2008b). Within Europe, eastern countries such as Poland and the Czech Republic are also becoming more important in extraction and processing activities.

Raw material prices and trends in markets

The costs of raw plant materials vary widely, and depend on many factors. For both pharmaceutical and cosmetic natural ingredients prices are influenced by quality factors and economic factors (CBI, 2008a; CBI, 2008b). Quality factors include the country of origin, the climate, the particular crop, the chemical profile of the plants and concentration of active ingredients and extraction methods. Economic factors are related to supply and demand, with supply being influenced by the size of the current crop, carry-over from previous crops, stocks held by traders, processors and final users and the availability of synthetic substitutes. In addition, documentation providing details of traceability and certification (e.g. Fair trade or organically certification) can influence pricing, as well as whether plants were cultivated or wild-collected.

Prices for pharmaceutical ingredients from plants are more stable when a constant supply can be guaranteed. With a fluctuating supply prices can vary greatly. Recent increases in the prices of certain raw materials in the EU have been caused by higher demand for the same ingredients to be used in other products, such as functional foods or cosmetics. For pharmaceuticals, raw ingredients usually make up only a small percentage (1-5%) of the cost of the final product. Processing of the raw material, for example extraction, accounts for 5-15% of the final cost. The rest of the price margin is from the final stages of the production process, when manufacturers make the final product (CBI, 2008b).

The price of herbal raw material collected in the wild depends very much on the demand for that material in the source country. In developing countries there is often an increasing demand, e.g. for medicinal plants as traditional medicinal practices become more formalized and mainstream (Cunningham, 1997). This competes for the demand for export and can cause prices to rise. In general prices depend largely on negotiations with traders, as well as on the specifications of the buyer. In the EU the large trading companies play a central role in determining the prices of raw materials.

For raw materials intended for use in the cosmetics sector, there is an almost direct relationship between the price of raw material and how much of it is used in the end-product (CBI, 2008a). Also the price of the final product will determine how much raw material can be used, as high-end products sold for high prices can use more expensive ingredients. Manufacturers have to stay within a certain target price to achieve the profit margin for a product, and formulators have to balance the amount of ingredients to achieve price targets and functional benefits (CBI, 2008a).

The prices of essential oils on the free market vary considerably, from relatively inexpensive oils produced on large scale, to expensive oils produced and traded in very small amounts (CBI, 2009a). Table 8 provides a summary of some indicative prices of essential oils to give an idea of typical prices. These include prices as low as €2/kg for orange oil to as high as $7500/kg for rose oil (personal communication Vassil Loutchev, Biogenic Stimulants Inc.). The market for essential oils lacks transparency, which makes determination of prices and margins difficult. A recent study estimated that the price of the final essential oil ingredient to be about five times more than the raw materials (CBI, 2009a). The value adding that occurs from raw material to final ingredient (essential oil or fraction of oil) is largely accounted for by the extraction process. High prices are also due to the large amounts of plant material typically needed to yield the oils. For example the production of 1 kg of rose oil requires 4000 kg of rose petals. Different grades of essential oil can be obtained from the same plant material. For instance ylang ylang oil is produced from the flower of *Cananga odorata* in a time-consuming three-phased distillation process. The oil drawn off from the first phase is of the best quality and is called ylang extra. Ylang II is drawn from the second phase and ylang III from the third phase. A blend of the three phases or the complete oil from the uninterrupted distillation is called ylang complete. The higher labor input, smaller yield and higher quality of the ylang extra oil is reflected in the price as compared to other grades (see Table 8).

It is possible to obtain some indicative prices of raw plant materials imported from various parts of the world. Care should be taken when comparing prices as the origins, chemical profiles and biological activities may vary considerably between similar products. It is also often difficult to obtain information on the cost price of the material, as opposed to the sales price. Brokers and traders are the main source of information on prices. A good source of a collection of indicative prices is the Market News Service for medicinal plants and extracts provided by the International Trade Center (ITC) in the form of a quarterly newsletter (the MNS bulletin, http://www.intracen.org/mns/welcome.htm). Apart from prices of raw materials and extracts consumed all over the world, it also provides information on supply and demand, trends in the industries involved, trade fairs and conferences related to these

industries. Table 9 is a summary of prices of selected raw materials from different parts of the world, with indicative prices per kilogram. From this summary it can be seen that the most expensive materials are often plant roots or rhizomes (e.g. ginseng, goldenseal rhizome). These plant parts are usually slow growing, and may take several years to reach maturity or be suitable for use as medicine. Parts of the plant that cannot be harvested non-destructively are typically more expensive than those that can (such as leaves). Rare plants with a limited geographical distribution are also more expensive, especially if their domestication and cultivation is difficult. Some plants may require very specific conditions to grow or to produce the desired active compounds. In some cases, even when cultivation of the material is possible, the wild material is considered superior or more potent. This is the case for ginseng roots and is reflected clearly in the price difference between wild and cultivated American ginseng.

With the aim of starting up a cultivation scheme of MAP material, looking at the prices of different kinds of material is one way of determining which are currently of higher value. Table 9 provides some prices for specific plants, and as discussed above, can give an indication of which plant species may be attractive for cultivation. It is also useful to know which plants are currently rare or endangered, as these may be very suitable for cultivation schemes. The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) website provides lists of all the plants currently considered endangered (http://www.cites.org/).

Another way to identify plants for a cultivation scheme is to predict and follow trends in the pharmaceutical and cosmetics industries concerning products, i.e. which ingredients are in high demand. In general, EU companies producing such products report a shortage of raw plant material, particularly for plant species not currently under cultivation (CBI, 2008b).

Table 8. Indicative prices of essential oils available on the market. Compiled from Market News Service (MNS) Medicinal Plants and Extracts Bulletin quarterly editions March 2008-September 2009.

Oil	**Species**	**Country of origin**	**€/kg**	**Year**
Rose	*Rosa damascena*	Bulgaria	5000[a]	2009
Sandalwood	*Santalum album*	Indonesia	1400	2009
Chamomile (Roman)	*Anthemis nobilis*	Hungary	700	2009
Chamomile (German)	*Matricaria recutita*	Hungary	750	2009
Chamomile (German)	*Matricaria recutita*	Egypt	550-600	2009
Cardomom	*Elettaria cardamomum*	Guatemala	150-220	2008
Cardomom	*Elettaria cardamomum*	Guatemala	20-25[b]	2009
Cinnamon bark	*Cinnamomum verum*	Sri lanka	155-230	2008
Ginger	*Zingiber officinale*	Sri Lanka	130-140	2008
Ginger	*Zingiber officinale*	India	70	2008
Rose geranium	*Pelargonium graveolens*	Egypt	125	2009
Geranium	*Pelargonium odorantissimum,*	China	80-90[c]	2009
Geranium	*Pelargonium odorantissimum,*	Egypt	100	2009
Ylang ylang extra	*Cananga odorata*	Comores	155-180	2009
Ylang ylang III	*Cananga odorata*	Comores	70-80	2009
Ylang ylang III	*Cananga odorata*	Indonesia	50	2009
Vetiver	*Chrysopogon zizanioides*	Indonesia	60-65	2009
Basil	*Ocimum basilicum*	India	40-45	2008
Basil	*Ocimum basilicum*	India	40-45	2009

Cumin seed	*Cuminum cyminum*	Iran, Egypt	60-70	2009
Lavender	*Lavandula angustifolia*	Bulgaria	50-70	2009
Lavender	*Lavandula angustifolia*	France	50-55	2009
Lavendin	*Lavandula hybrida*	France	20-25	2009
Tea trea	*Melaleuca alternifolia*	Australia	37	2009
Nutmeg	*Myristica fragrans*	Indonesia	40-60	2009
Orange (bitter)	*Citrus aurantium*	Italy	45-50	2009
Bergamot oil	*Citrus aurantium* subsp. *bergamia*	Sicily, Italy	40	2009
Lemon	*Citrus limon*	Argentina and Spain	30-50	2009
Lime	*Citrus aurantifolia*	Mexico	20-25	2009
Palmarosa	*Cymbopogon martinii*	India	25-35	2009
Peppermint	*Mentha piperita*	USA	40-45	2009
Peppermint	*Mentha piperita*	India	30-35	2009
Peppermint	*Mentha piperita*	China	9	2009
Peppermint, dementholised	*Mentha piperita*	India	7	2009
Spearmint	*Mentha spicata*	China	18-20	2009
Spearmint	*Mentha spicata*	India	18-20	2009
Arvensis mint	*Mentha arvensis*	India	9-10	2009
Menthol	*Mentha spp.*	China	13	2009
Menthol	*Mentha spp.*	India	11-12	2009
Patchouli	*Pogostemon cablin*	Indonesia	25-30	2009
Litsea cubeba	*Litsea cubeba*	China	17-18	2009

Litsea cubeba	*Litsea cubeba*	Australia	20	2009
Cinnamon leaf	*Cinnamomum verum*	Sri lanka	10-12	2009
Cassia bark	*Cinnamomum cassia*	China	15	2009
Eucalyptus, high cineole	*Eucalyptus spp.*	Australia	30-35	2009
Eucalyptus, high cineole	*Eucalyptus spp.*	Australia	15	2009
Eucalyptus, high cineole	*Eucalyptus spp.*	China	6 -7	2009
Eucalyptus, high citronellal	*Eucalyptus citriadora*	China	16	2009
Eucalyptus, high citronellal	*Eucalyptus citriadora*	Brazil	7-9	2009
Lemongrass	*Cymbopogon citratus*	India	10-12	2009
Citronella	*Cymbopogon winterianus*	Sri Lanka	15-16	2009
Citronella	*Cymbopogon winterianus*	China	6-8	2009
Citronella	*Cymbopogon winterianus*	Indonesia	6-8	2009
Anis seed	*Pimpinella anisum*	China	7-9	2009
Star anise	*Illicium verum*	China	6-8	2009
Orange (sweet)	*Citrus sinensis*	Brazil	2-4	2009
Clove leaf	*Syzygium aromaticum*	Indonesia	5-6	2008

[a] exchange rates of December 2009, [b] sold per ton, [c] sold per drum

Table 9. Summary of indicative prices of raw materials from different parts of the world (Summarized from Market News Service quarterly Newsletters "Medicinal Plants and Extracts" March 2008-September 2009). Prices in $/kg unless otherwise indicated.

AFRICA

Product	Botanical name	Grade	Cult/wild	Price($)/kg	Origin	Year
Buchu leaf	*Agathosma betulina*	PhFr	cultivated	56.00	South Africa	2010
Vanilla fruit	*Vanilla planifolia*	NK	cultivated	20.22-25.27	Madagascar	2010
Myrrh powder	*Commiphora spp.*	NK	Wild	11.00-15.00	Africa	2009
Pygeum bark	*Prunus africana*	NK	Wild	8.80-12	Africa	2009
Gotu kola herb	*Centella asiatica*	PhEur	Wild	11.70	Madagascar	2009
Spearmint leaf	*Mentha spicata*	PhFr	cultivated	7.34	Egypt	2010
Clove flower bud	*Syzygium aromaticum*	NK	cultivated	5.84	Madagascar	2010
Fennel fruit	*Foeniculum vulgare*	NK	cultivated	1.98	Egypt	2010
Rose hip	*Rosa canina*	NK	Wild	2.60	South Africa	2008
Rooibos herb	*Aspalathus linearis*	NK	cultivated	€1.80	South Africa	2009
Rosemary herb	*Rosmarinus officinalus*	NK	Wild	1.39	Morocco	2009

China and other east Asian countries

Product	Botanical name	Grade	Cult/wild	Price($)/kg	Origin	Year
Asian Ginseng root	*Panax ginseng*	Medium	cultivated	61.73	China	2010
Schisandra fruit	*Schisandra chinensis*	NK organic	Wild	48.50	China (North)	2010
Schisandra fruit	*Schisandra sphenanthera*	NK	Wild	19.80	China (South)	2010
Schisandra fruit	*Schisandra sphenanthera*	NK	Wild	1.90-2.50	Sichuan, China	2009
Ginkgo dry extract*	*Ginkgo biloba*	standardized	NK	23.00-25.00	China	2010

Lycium fruit	*Lycium barbarum*	NK, organic	Wild	26.45	China	2009
Lycium fruit	*Lycium barbarum*	NK	Wild	11.00	Shaanxi	2009
Safflower	*Carthamus tinctorius*	PPRC, organic	cultivated	16.00	China	2010
Eleuthero root	*Eleutherococcus senticosus*	NK	Wild	8.80	Jilin	2009
Ginger whole peeled	*Zingiber officinale*	NK	cultivated	3.09	China	2010
Garlic bulb	*Allium sativum*	NK	cultivated	2.05	China	2010

India and other Asian countries

Product	**Botanical name**	**Grade**	**Cult/wild**	**Price $/kg**	**Origin**	**Year**
Saffron style and stigma	*Crocus sativus*	Organic	cultivated	3240.00-4780.00	Kashmir	2010
Shatavari root	*Asparagus racemosus*	NK	wild/ cultivated	81.37	India	2008
Andrographis herb	*Andrographis paniculata*	NK	wild/ cultivated	61.02	India	2008
Costus root	*Saussurea costus*	NK	wild	65.10-71.20	India	2008
Nirvisa root	*Delphinium denudatum*	NK	NK	20. 40	Nepal	2008
Cardomom seed	*Elettaria cardomomum*	NK	cultivated	11.80	India	2009
Cassia bark	*Cinnamomum aromaticum*	NK	Wild/ cultivated	1.57-8.44	India	2009
Rauvolfia root	*Rauvolfia serpentina*	NK	NK	7.67-8.95	India	2008
Clove flower bud	*Syzygium aromaticum*	NK	cultivated	7.00	India	2009
Chirata herb	*Swertia chirayita*	NK	wild	6.10-8.14	India	2008
Nutmeg kernel	*Myristica fragrans*	NK	cultivated	6.85	India	2009
Psyllium husk	*Plantago ovate*	Organic	cultivated	7.94	India	2009

Psyllium husk powder	*Plantago ovate*	NK	cultivated	6.96	India	2009
Psyllium husk 99%	*Plantago ovate*	NK	cultivated	5.10	India	2009
Psyllium husk 98%	*Plantago ovate*	NK	cultivated	4.60	India	2009
Psyllium husk 95%	*Plantago ovate*	NK	cultivated	4.00	India	2009
Psyllium husk 85%	*Plantago ovate*	NK	cultivated	3.60	India	2009
Ginger rhizome	*Zingiber officinale*	NK	cultivated	2.50-2.64	India	2009
Ashwagandha root	*Withania somnifera*	NK	cultivated	1.50-1.63	India	2009
Fennel fruit	*Foeniculum vulgare*	NK	cultivated	1.99	India	2010
Turmeric rhizome	*Curcuma longa*	NK	cultivated	0.96-1.40	India	2009
Senna pod	*Cassia angustifolia*	Organic	cultivated	0.77	India	2010
Senna leaf	*Cassia angustifolia*	Organic	cultivated	0.72	India	2010
Psyllium seed	*Plantago ovata*	NK	cultivated	1.00-1.10	India	2009
Coriander fruit	*Coriandrum sativum*	NK	cultivated	1.00-1.19	India	2009
Bacopa herb	*Bacopa monnieri*	NK	wild	0.70-0.80	India	2009
Amla fruit	*Phyllanthus emblica*	NK	wild	0.60-0.70	India	2009
Fenugreek seed	*Trigonella foenum-graecum*	NK	cultivated	0.90	India	2010
Garlic bulb	*Allium sativum*	NK	cultivated	0.56	India	2009

North America

Product	**Botanical name**	**Grade**	**Cult/wild**	**Price $/kg**	**Origin**	**Year**
Ginkgo dry extract*	*Ginkgo biloba*	USP	NK	175.00-275.00	USA	2010
American ginseng root	*Panax quinquefolius*	Hong Kong Top	wild	110.00	USA	2010

American ginseng root	*Panax quinquefolius*	Medium	cultivated	88-110.00	Minnesota	2010
American ginseng root	*Panax quinquefolius*	USP	cultivated	46.00	Canada	2010
American ginseng root	*Panax quinquefolius*	small legs	cultivated	44.00	Minnesota	2010
American ginseng root	*Panax quinquefolius*	USP	cultivated	47.00	Wisconsin	2010
Goldenseal rhizome	*Hydrastis canadensis*	Extract quality	cultivated	80.00	Wisconsin	2010
Goldenseal rhizome	*Hydrastis canadensis*	NK	wild	64.00	USA	2010
Black cohosh rhizome	*Actaea racemosa*	NK	wild	16.0-20.0	USA	2009
Black cohosh rhizome	*Actaea racemosa*	Extract quality	wild	11.65	USA	2010
wild cherry bark	*Prunus serotina*	Organic	wild	11.95	USA	2008
Cardomom seed	*Elettaria cardamomum*	Organic	cultivated	24.80-28.50	Guatemala	2008
Cranberry fruit powder	*Vaccinium macrocarpon*		cultivated	25.00	USA	2008
Echinacea root	*Echinacea purpurea*	PhEur, organic	cultivated	12.15	Pacific NW	2010
Echinacea root	*Echinacea purpurea*	USP, organic	cultivated	20.90	California	2010
Echinacea herb	*Echinacea purpurea*	PhEur, organic	cultivated	5.95	Pacific NW	2010
Slippery elm bark	*Ulmus rubra*	USP, organic	wild	28.66	USA	2010
Saw palmetto fruit	*Serenoa repens*	USP	wild	6.61	Florida	2010
Peppermint leaf	*Mentha piperita*	PhEur , organic	cultivated	7.16-8.16	USA	2010
Coriander fruit	*Coriandrum sativum*	extract quality	cultivated	1.60	Canada	2010
Cascara sagrada bark	*Frangula purshiana*	NK	wild	10.60-11.00	USA	2009
Wild yam root powder	*Dioscorea villosa*	NK	wild	7.90	USA	2008

Wild yam root, fermented	*Dioscorea composita*	NK	wild	9.50	Mexico	2008

South America

Product	Botanical name	Grade	Cult/wild	Price $/kg	Origin	Year
Maca hypocotyle	*Lepidium meyenii*	NK	cultivated	12.00-15.00	Peru	2008
Cat's claw stem bark	*Uncaria tomentosa*	NK	wild/ cultivated	9.50	Peru	2008
Stevia leaf	*Stevia rebaudiana*	NK	cultivated	10.70-12.00	Paraguay	2009
Mate leaf	*Ilex paraguariensis*	organic	cultivated	8.8-11.00	Argentina, Brazil	2009
Pau d'arco bark	*Tabebuia impetiginosa*	NK	wild	5.60	Argentina, Brazil	2009
Cha de bugre leaf powder	*Cordia salicifolia*	NK	not known	2.80	Brazil	2009
Mate leaf powder	*Ilex paraguariensis*	NK	cultivated	2.76	Brazil	2009
Night-blooming cereus powder	*Selenicereus grandiflorus*	NK	NK	2.75	Brazil	2009
Muira puama bark powder	*Ptychopetalum olacoides*	NK	wild	2.61	Brazil	2009

Europe

Product	Botanical name	Grade	Cult/wild	Price $/kg	Origin	Year
Narrow-leaved comeflower root	*Echinacea angustifolia*	NK	Wild/cultivated	58.90	USA	2008
Bilberry fruit	*Vaccinium myrtillus*	NK	wild	16.24-24.98	Eastern Europe	2009
Bilberry fruit	*Vaccinium myrtillus*	organic	wild	29.01	Bosnia Herzegovina, Croatia, Poland	2009
Dandelion root	*Teraxacum officinale*	NK	wild	6.55-7.92	Eastern Europe	2009
Dandelion root	*Teraxacum officinale*	organic	wild	14.35	Croatia, Poland	2009
Elder flowers	*Sambucus nigra*	NK	wild	7.78-9.282	Eastern Europe	2009

Elder flowers	*Sambucus nigra*	organic	wild	13.66	Hungary	2009
Elder flowers	*Sambucus nigra*	organic	wild	14.34	Bosnia Herzegovina	2009
Elder fruit	*Sambucus nigra*	NK	wild	6.62	Eastern Europe	2009
Elder fruit	*Sambucus nigra*	organic	wild	14.96	Croatia, Poland	2009
Fenugreek seed	*Trigonella foenum-graecum*	NK	cultivated	1.15	Turkey	2009
Mugwort herb	*Artemisia vulgaris*	organic	wild	2.12	Hungary	2009
Purple coneflower	*Echinacea purpurea*	NK	cultivated	9.00	UK	2008
Ramsons herb	*Allium ursinum*	organic	wild	6.825-8.19	Bosnia Herzegovina /Hungary	2009
Stinging nettle herb	*Urtica dioica*	organic	wild	2.12	Hungary	2009
Caraway fruit	*Carum carvi*	NK	cultivated	2.90	Netherlands	2008

NK: not known, PhEur: European Pharmacopoeia standards, PhFr: French Pharmacopoeia standards, USP: United States Pharmacopoeia, PPRC: Pharmacopoeia of Peoples' Republic of China standards. *Price of extract, as price information of raw leaf material not available.

Trends in the markets: Medicinal plants

In the last few years much attention has been focused on products aimed at improving mental abilities and cognitive functions (CBI, 2008b). For instance, there have been large increases in products containing *Gingko biloba*, ginseng and St. John's wort. For the future it is believed that plant-based "herbal medicines" with cancer preventative or combating properties will be very popular. Another group of products currently high in demand is those that help relieve stress and fatigue, and this type of product is predicted to remain very popular. The CBI market survey on the market for natural ingredients for pharmaceuticals in the EU (CBI, 2008b) listed some specific ingredients identified as being increasingly popular in product innovations and used in pharmaceutical products. These were blackcurrant and other anthocyanin-containing berries and fruits (for supplements with antioxidant properties), Echinacea (for cold and flu remedies), green tea extract (for various products, mostly for antioxidants), Devil's claw (*Harpagophytum procumbens*, for remedies against arthritis), *Artemisia annua* (for anti-malarial remedies), the alkaloid quinine (used in various products) and vitamins from natural sources, as opposed to synthetically produced vitamins.

Another way to predict future demand for raw medicinal plant material is to look at clinical trials of medicinal plants from recent years or currently in progress. A list providing the number of clinical trials of medicinal plants in 2007 was reported in the CBI market survey (CBI, 2008b). The top ten plants for that year were St John's wort (177 trials), Ginkgo (166), garlic (101), Licorice (81), Saw palmetto (66), Ginseng (Korean or American) (59), Cone flower (47), Valerian (42), Kava (39) and Ginger (38). For each of these plants a number of patent applications were filed regarding some aspect of their medicinal use. The most patents were related to ginseng, with 53 patents issued in 2007 (CBI, 2008b). From this one is able to get an idea of ingredients in demand. A precise picture is less easily obtained, however, because the pharmaceutical industry and ingredients markets are by nature very closed.

Trends in the markets: Cosmetics

In the EU the trend is for cosmetics manufacturers to deal with specialized ingredient suppliers, who are more and more interested in organic production of raw materials (CBI, 2008a). Another trend concerning raw MAP materials for the cosmetics industry is the decline of large-scale cultivation of lower-value products in the EU, and an increased importation of such material. Production of more specialized, high-value plant material is increasing, in particular by organic or biodynamic cultivation methods. In the EU cosmetics industry there are increasing requirements for documentation and safety assessment of cosmetics ingredients and products, as traceability of the entire

value chain becomes more important. Legislative issues and documentation will be discussed in section 6.

In general the demand for natural ingredients for cosmetics is increasing (CBI, 2008a). Consumers find cosmetic products containing naturally derived ingredients more and more attractive, in particular if they also have a functional benefit. Demand for this category of therapeutic cosmetics or "cosmaceuticals" is predicted to grow. In the EU cosmetics manufacturers are continuously searching for new products and ingredients. Exotic materials from developing countries often have interesting properties, but do not have the necessary safety and technical documentation required by the industry. Also a lack of reliable supply chains is a problem with such materials. This could be a good entry point for growers in the EU to grow high quality exotic MAP crops.

Another trend currently observed is the increasing use of food ingredients in cosmetic products. Examples are cocoa, rice, fruit pulp, and seed oils (e.g. kernel or seed oil from grapes, mango, blueberry, papaya and pomegranate). Also essential oils from citrus fruit peels have become very popular. With essential oils from plants, the whole oil is used if the desired compound makes up 70-80% of the oil. If not it is also common to use a fraction of the oil to obtain a higher concentration of the desired compound(s). There is interest in many different essential oils, many from tropical origins. There are opportunities for greenhouse growers to produce plants that are otherwise not suitable for the European climate. For cosmetic products, essential oils are primarily used for their fragrance properties. However, certain essential oils are known to possess other interesting properties, e.g. antibacterial or antifungal, and such oils may be very promising (e.g. allowing use of less preservatives in a product or for use in an anti-dandruff product). Another trend is to produce cosmetics that are fully organic. To produce more such products, there is a need for ingredients that have a certain function in the product, such as emulsifiers and preservatives. The demand for such ingredients derived from organically grown crops is expected to grow significantly.

Trends in the market: Plant protection products

In many parts of the world laws and policies recently implemented a move towards the use of safer agrochemicals and away from harmful synthetic ones. Integrated Pest Managament (IPM) programs are increasingly being implemented at regional and national levels. Natural pesticides are suitable for use in such programs. There is also a large demand in the organic agriculture sector, and in the developed world this is a promising, expanding market. The synthetic pesticide market is expected to decline by 1.5% per year, while the biopesticide market is expected to keep growing to be worth more than a billion dollars in the next few years (Thakore, 2006).

New plant protection products are being searched for (Guleria and Tiku, 2009). Screening is often guided by traditional use of plants to protect crops. To avoid the lengthy process of setting up new cultivation schemes for plants, those that are already available on a large scale should be investigated for potential new products. The use of agricultural waste products to produce plant protection products is particularly attractive. For example after the juice has been extracted from citrus fruit, oils can be extracted from the leftover peels. These oils possess various biological activities, including insect repellant, insecticidal and antimicrobial (Shahidi and Zhong, 2005). The method of extraction should ideally be environmentally friendly.

The growth of this sector is expected to continue, but a factor that may constrain growth is the rapidly changing and demanding regulations involved in the registration and release of new products (Isman, 2006). In industrialized countries large investments are needed to cover regulatory costs (Isman, 2006). In the United States essential oil-based pesticides are exempt from registration if the oil is from a plant used in food or beverages. This will help the commercial development of such products (Guleria and Tiku, 2009). A further obstacle to overcome in this market is growers' lack of confidence in new plant-derived pesticides. Often these kinds of products have slower actions than conventional pesticides, and efficacy may not be immediately visible. This may lead to reluctance on the growers' side to buy new products (Isman, 2006). Slower actions may be less critical to people who use pesticides in and around their homes (e.g. ornamental plants, vegetable gardens). Safer alternatives to synthetic pesticides for this role may be the most important market for plant-based pesticides, especially in developed countries (Isman 2008).

There is increasing interest in essential oils as novel insecticides. For example mixtures of mono- and sesquiterpenes have promising spatial and contact repellant properties (Zhu et al. 2006). Other compounds showing promise but that have not yet been commercialized are acetogenins (from *Annona squamosa*, *A.muricata*) as slow-acting stomach poisons (Alali et al., 1999). Many compounds or extracts have been shown to have antifeedant properties, but none of these have been commercialized (Isman 2006). Problems are related to behavioral plasticity of insects and the complexity of ecological interactions in a field situation. For instance different species may respond differently to an antifeedant, so that one species may be deterred and another may be attracted (Isman 2006).

Trends in the market: Essential oils

In the flavors and fragrances market, the market for essential oils and extracts is predicted to grow the fastest, at more than 5% per year (CBI, 2009b). Fruity flavors in

food and beverages are currently very popular, and are believed to be driven by consumers wanting healthy and natural products. This should contribute to an increased industrial demand for fruity essential oils. An increasing demand for natural and organic flavors is requiring manufacturers to use compatible production processes. Apart from an increasing demand for essential oils produced from organically-grown plants, there is also a growing need for fractionated oils. Fractionation is a separation process that allows the isolation of individual or groups of compounds from a complex essential oil mixture. Applying fractionation to organic essential oils does not change their status as organic, and in this way individual compounds responsible for a particular fragrance can be obtained and combined with others to obtain a wide range of new organic flavors and fragrances (CBI, 2009b). Fair trade certified oils are also becoming more popular.

In the EU the market for natural colors and flavors is consolidating, and many flavor houses have been taken over by large pharmaceutical and chemical companies (CBI, 2009b). This allows them access to more laboratory facilities and research activities, which allows flavor houses to develop new products. The consolidation also causes flavor manufacturers to require larger raw material supplies. The larger companies have more buying power and prefer to shorten supply chains. For large companies the ideal situation is to have a strong relationship with exporters that can provide consistent supplies in large amounts. Small and medium-sized manufacturers continue to obtain raw material from smaller suppliers. The location of essential oil production is not so important to manufacturers, as worldwide sourcing is favored due to relatively small volumes of the product being traded. Transportation costs are not prohibitively high. For this reason, raw materials produced outside the EU continue to be imported.

Trends in the market: Dyes and colorants

The market for natural fabric dyes consist of on the one hand hobbyists using them on a small scale and on the other hand large-scale industrial manufacture for large retailers. For the larger scale use, it is most important that the natural dyes are of consistent quality from batch to batch. Also the ability to provide a stable supply is essential for larger scale users. Dye production processes have to be standardized. Thus far large-scale producers have been reluctant to use natural dyes due to issues with supply and inconsistency of color and quality.

Plant-derived food and cosmetics colorants will be in increasing demand as manufacturers continue to look for alternatives to synthetics. The cost of natural colorants is still two to ten times higher than synthetic colorants. The demand from consumers is strong as more natural ingredients are desired in the products they use. The natural food color market is doing very well, and further positive developments are

expected as research into the stabilization of natural color compounds advances (CBI, 2009b).

Expectations from industry in terms of quality of raw material.

In the EU various rules and regulations exist that apply to the production of and trade in raw plant material intended for industrial use. Some requirements are set by national or regional legislation. Additional requirements can also be set by the companies involved, particularly manufacturers of the end products using the plant material. It is important to know which buyers are aimed at when producing plant material, as this will determine requirements that need be met. For growers wanting to produce an existing crop for a new use, this will also help to determine whether any current agricultural practices need to be changed. The European Commission Directorate General Health and Consumers has a Pharmaceuticals unit, which provides all the necessary information about legislation in this sector on the following website: http://ec.europa.eu/health/human-use/index_en.htm . Similarly, for the cosmetics sector, the following website provides information about various aspects of the sector: (http://ec.europa.eu/consumers/sectors/cosmetics/index_en.htm#top).

In the cosmetics and pharmaceutical industries, it is highly desirable that raw materials from cultivated plants are produced using Good Agricultural Practice (GAP). It is currently not a legislative requirement, i.e. neither national laws nor EU law states that GAP should be used. However, most companies using raw plant materials will not accept cultivated material not produced in this way. The cultivation of plants according to GAP means using established standard operating procedures (SOPs) that will ensure good quality and safety, and thoroughly documenting all actions and procedures to ensure complete traceability. Initially GAP recommendations were set up for food crops, but recently additional ones have been formulated for medicinal plants cultivated as crops. In 1998 the European Herb Growers Association (Europam) released recommendations on GAP (EUROPAM, 1998). The World Health Organization (WHO) published guidelines on Good Agricultural and Collection Practices (GACP) in 2003 (WHO, 2003). After releasing three drafts of "Points to consider on GACP" in 1999, 2002 and 2005, the European Medicines Agency (EMEA) Herbal Medicinal Product Working Party published a document "Guidelines on GACP for starting materials of herbal origin" in 2006 (EMEA/HMPC, 2006; EMEA/HMPWC, 1999; EMEA/HMPWC, 2005; EMEA/HMPWP, 2002).

Any process or procedure used in the production of a pharmaceutical product must be done according to Good Manufacturing Practice (GMP). This is compulsory according to EU legislation (Commission Directive 91/356/EEC) as well as national legislation of

member states. This legislation is also relevant for any extracts or other value-added plant products for human use. This directive and further guidelines and revisions added since are summarized in "EU Guidelines to Good Manufacturing Practice for human and veterinary use" published by the European Commission in 2008 (EC, 2008).

For cosmetics it is currently not compulsory to use GMP, however most companies do use cosmetics-specific GMP procedures in the manufacture of products. The Cosmetics directive 76/768 of 1976 has been amended and adapted many times since its implementation. A new directive is now being set up and will be in place in 2010. This legislation is aimed at products, not for raw materials and ingredients. A useful database is the EC database for Cosmetics Ingredients and Substances (http://ec.europa.eu/consumers/cosmetics/cosing/). Here one can search for a substance and find information on its relevance for the cosmetics industry, e.g. whether it is a banned substance or not, or for what kind of products it is used (e.g. fragrance, coloring, sun filter etc.).

REACH is a new European Community Regulation on chemicals and their safe use (EC 1907/2006). It deals with the **R**egistration, **E**valuation, **A**uthorization and Restriction of **Ch**emical substances. The new law entered into force on 1 June 2007, and may have some implications for ingredients isolated from plants, including extracts and essential oils. See: http://ec.europa.eu/environment/chemicals/reach/pdf/2007_02_reach_in_brief.pdf for a brief outline.

Apart from requirements set in legislation, companies may have certain additional requirements or expectations from the raw material they use to make their products. These expectations are mostly met by obtaining certification from an independent organization stating that the grower or producer has followed certain guidelines. For wild-collected plants many certifications apply, such as Sustainable Forest Management certification from the Forest Stewardship Council (http://www.fsc.org/).

For cultivated plants, a common requirement is organic certification. This means that a crop was produced without input of synthetic agrochemicals, and that the farmland used has been free of such chemicals for a certain number of years, that the growing site was inspected and the whole growing process audited. Different countries have different organizations dealing with organic certification, while the International Federation of Organic Agriculture Movements (IFOAM), an NGO, is a main international organization (www.ifoam.org).

Social certification may be relevant to the cultivation of MAPs, depending on where it occurs. The aim of social certification is to ensure that trade is based on fair and ethical standards. For example if plants are cultivated in a developing country for a company in the EU this certification ensures states that producers and workers are given fair terms of trade and fair prices and wages. Examples of social certification systems include those of the Fair Trade Labeling Organization International (FLO) (www.fairtrade.net), International Fair Trade Association (www.ifat.org), and of Social Accountability International (SAI) (http://www.sa-intl.org).

The International Organization for Standardization (ISO) is an international body that also establishes quality standards. Two standards are relevant to the production of MAPs. ISO 9001:2001 for quality management systems was implemented to ensure quality requirements are satisfied and to enhance satisfaction in supplier-consumer relationships. An updated new standard (ISO 9001:2008) is now gradually being implemented for quality management systems. A second standard, ISO 14001:2004 is for environmental management systems, and is relevant for organizations that wish to operate in an environmentally sustainable way (www.iso.org).

Conclusions

This review briefly described some trends in industries that use fine chemicals derived from plants, which could help growers choose an interesting plant species to cultivate. The indicative prices give some guidance as to whether the material can be produced in an economically feasible way. Knowing what the requirements are from the various industries that use plants as raw materials is also very important, as this may affect the agricultural practices used. Where more information is needed on any of these issues, the websites provided can be a good starting point.

There are several approaches to find a buyer for cultivated plant material. The first is through an agent, trading house or distributor of plant material. Such companies obtain plant material from various sources (growers and wild collectors) and sell them on to other companies (manufacturers/extraction companies). The second is to find a direct buyer on open markets (for example online at www.greentrade.net or www.ingridnet.com) by advertising material for sale or by searching for buyers of material. Another approach is to become a contract cultivator for companies (e.g. pharmaceutical and cosmetic companies) that use the raw materials. Such companies usually want to have control of the whole chain from raw material to end product. To set up such a supply chain, collaboration with the company is essential from the early R&D stages. This is necessary so that the important steps can be optimized from the start on a small pilot scale, e.g. cultivation practice to ensure constant compositions,

time of harvest, post-harvest treatment. Thereafter a reproducible source of biomass can be established so that the supply chain and production processes can be further developed. Different buyers will have different expectations and requirements, but some points are relevant for all MAP crops:

Efficacy; the compound/extract must work in practice (in the hands of the end user) and not just in a laboratory. Plant material cultivated for a specific compound or group of compounds should be standardized, i.e. cultivated in a way that the level of the desired compound is known.
Safety; Increasing requirements are established to prove that plant material and products are safe for the consumer and/or environment. These requirements exist at a company, national and regional level.
Traceability; In order to guarantee efficacy and safety all steps in the production of plant material should be well documented. Good traceability benefits the producer and buyer of MAP material.
Supply; Sufficient amounts and a constant supply of raw material are very important for establishing good trade relationships.
Demand; Producers should keep themselves informed of ever changing demands for MAP material.

Many plant species are threatened due to overharvesting for medicinal or other use. There is great need to protect plant diversity. There is also a need to develop more sustainable ways of obtaining industrial products from renewable resources. The cultivation of MAPs for industrial products can address these issues.

Chapter 2. NMR-based metabolomics analysis

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Abstract

In recent years the trend in biological research has been towards a more holistic, or "systems" approach to observing living organisms. Systems biology aims to do this by observing all the components of living organisms and how they interact. Studying the full complement of DNA (genomics), expressed RNA (transcriptomics) or proteins (proteomics) are important parts of systems biology. The study of all the small metabolites in a living organism is called metabolomics, and represents the most recent addition to the systems biology. Together with computational tools, this approach allows for new insights into how cells, tissues and organisms function. While the ultimate goal of metabolomics (analyzing and quantifying all metabolites in a given sample) is not yet feasible, several analytical platforms make it possible to achieve this goal to varying degrees. In this chapter the concept of metabolomics is introduced, and different analytical profiles are compared. NMR-based metabolomics is reviewed in more detail, with a focus on plants as the subject of study. Technical aspects of NMR analysis, data analysis and sample preparation is discussed, as well as examples of some practical applications of NMR metabolomics in plant research.

Introduction

From chapter 1, several aspects of medicinal and aromatic plants as raw material for industrial use emerged as important for the quality of such products. The most important is the consistent level of active compound(s) for which the plant is cultivated. In establishing a new industrial crop it is important to have the tools to monitor the target metabolites in the plant. These tools can be used to optimize cultivation practices in the field (e.g. fertilizer treatments), determine optimal time of harvest, as well as establish good post-harvest protocols (e.g. drying and storage conditions). In this thesis, NMR-based metabolite profiling was chosen as the main analytical tool for analyzing medicinal plant material, and will be introduced in this chapter.

A big shift in biology in relatively recent years was the move away from pure reductionism and towards a more holistic or systems approach. "Systems biology" recognizes that organisms are best understood by observing all their components and their interactions (Cowley, 2004). Genomics, transcriptomics and proteomics involve the study of the entire DNA sequence (genome), set of RNA transcripts (transcriptome) or set of proteins expressed (proteome) by an organism at a particular time, respectively. Similarly metabolomics involves the study of the metabolome, the entire complement of small metabolites in a given cell, tissue or organism (Oliver et al., 1998; Sumner et al., 2003). With computational tools these "-omics" approaches can be integrated to reveal new insights on the functioning of living things. In spite of technological advances, the aim of metabolomics (to identify and quantify all metabolites) is still not entirely feasible. No single analytical technique or combination of techniques is sensitive, selective or comprehensive enough to measure all metabolites in a cell or tissue (Weckwerth, 2003). This is due to the large number and the great diversity in structure, chemical properties, concentration, and stability of metabolites in any given sample (Verpoorte et al., 2008). Until now (and in this chapter) studies referring to "metabolomics" in fact may be more accurately described as "metabolite fingerprinting" and "metabolite profiling". By definition, metabolite fingerprinting is high throughput qualitative screening of metabolic composition to perform discrimination analysis and comparison of different samples, for example two different genotypes. Metabolite fingerprinting is often followed by metabolite profiling which involve identification and quantitation of selected and limited number of metabolites.

Even though the full complement of metabolites cannot be measured yet, a metabolomics/metabolite profiling approach can be very useful in biological studies. In a sense metabolomics works on a level closest of all the "-omics" to an organism's phenotype. This is useful in studying medicinal plants for instance, where the bioactive

metabolite(s) of interest needs to be understood in terms of the rest of the plant's biology.

Platforms for Metabolomics

The ideal analytical technique for metabolomics should be unbiased, rapid, reproducible, and stable over time, while requiring only simple sample preparation (Verpoorte and Kim, 2010). Different platforms are available for metabolome analysis including high performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), capillary electrophoresis-mass spectrometry (CE-MS), Fourier transform ion cyclotron-mass spectrometry (FT-MS), and nuclear magnetic resonance (NMR). Among these, GC- or LC-MS, and NMR are most widely used. Each of these has advantages and disadvantages and there is always a compromise in terms of sensitivity, high throughput, robustness, quantitative accuracy, and suitability for specific chemical classes of metabolites. Nevertheless, a carefully chosen analytical method can be an excellent initial strategy for gaining a first impression of a metabolic profile which can ultimately be used to identify key biochemical leads to further or more focused studies (Hirai et al., 2004)(Hirai et al., 2005). A brief description of the main platforms used for high throughput analysis of metabolites is given below followed by a more detailed description of NMR.

Mass Spectrometry (MS)

Mass Spectrometry is one of the primary detection methods for metabolomics due to its high sensitivity, speed and broad application. Many papers have shown its suitability for metabolite detection in complex matrices (Fiehn et al., 2000; Fiehn, 2002). Gas chromatography (GC) or liquid chromatography (LC) is commonly used for metabolite separation prior to MS detection. These combinations of different separation techniques with MS along with their applications have been extensively discussed by Dettmer *et al.* (Dettmer et al., 2007). The following sections present brief accounts on each of these techniques and their advantages and disadvantages.

Gas Chromatography–Mass Spectrometry (GC-MS)

This technique is the most popular and widely applied method in metabolomics. Its popularity is mainly due to robustness of both separation and detection along with the availability of some excellent metabolite databases to aid identification. This technique combines high sensitivity and resolution with a reproducible fragmentation pattern of the separated molecules. Application of two-dimensional GC-MS has resulted in further improvement in resolution (Dallüge et al., 2003; Vial et al., 2011). GC-MS is the principal technique for separation and detection of metabolites that are naturally volatile

at temperatures up to 250 °C (e.g. fatty acids, aliphatic alcohols and esters essential oils) at the cost of thermolabile compounds. The technology can also be applied to groups of non-volatile, polar (mainly primary) metabolites, such as amino acids, sugars and organic acids, by converting these into volatile and thermostable compounds through chemical derivatization. These derivatized samples can then be analyzed by GC-MS and detailed information on many of the key primary metabolites in plants can be obtained in a single chromatographic process (Roessner et al., 2001; Desbrosses et al., 2005). However, the extent of derivatization or incomplete derivatization can cause the problem of more than one peak for the same compound (Ryan and Robards, 2006). Comparison between chromatograms of identical peaks is possible but absolute quantitation requires calibration curves of each compound. Another limitation of this technique is that complex plant secondary metabolites, like phenolic glycosides, cannot be analyzed by gas chromatography.

Liquid Chromatography-Mass Spectrometry (LC-MS)

LC-MS is a very important and versatile technology in metabolomics, which can be used for the analysis of many plant metabolites without any chemical derivatization. Advances in chromatographic technologies (like ultra-performance liquid chromatography) together with advances in column chemistry (like hydrophilic interaction chromatography and long monolithic columns) resulted in a significantly improved separation potential. The technology is inherently restricted to molecules which can be ionized, either as positively or negatively charged ions, before moving through the MS. The wide range of analytes in terms of molecular weight and polarity along with precise molecular weight determination are certainly the strong points of LC-MS (Sumner et al., 2003; Gobey et al., 2005). An authoritative review on LC-MS in plant metabolomics has been recently published (Allwood and Goodacre, 2010). Unlike GC-MS, mass spectral libraries or databases for LC-MS are limited, so that metabolite identification remains a challenge in this platform.

Capillary Electrophoresis- Mass Spectrometry (CE-MS)

The recent development of capillary electrophoresis (CE) as an alternative separation technology, particularly for charged metabolites, is growing in popularity particularly when combined with MS for extra selectivity and sensitivity (Soga et al., 2003). High-resolution chromatographic separation and sensitive detection of water soluble extracts make a strong combination suitable for the analysis of a diverse range of polar and water soluble primary and secondary metabolites (Sato et al., 2004). Derived from CE, capillary electro chromatography (CEC) is another promising separation technique. It uses LC or has monolithic stationary phases hence a hybrid of liquid chromatography and capillary electrophoresis. The combination of CEC with MS, the interfaces used,

and different bio-analytical applications like analysis of proteins, peptides, amino acids, and mixture of saccharides, has been reviewed by Klampfl (Klampfl, 2004).

Fourier Transform Ion Cyclotron Resonance-Mass Spectrometry (FT-ICR-MS)
A relatively less applied technique known as Fourier transform ion cyclotron-mass spectrometry, often called as FT-MS, is capable of non-targeted metabolic analysis and is suitable for rapid screening of similarities and dissimilarities in large collections of biological samples, e.g., plant mutant populations (Cooper and Marshall, 2001). After a pause following the first paper on this topic by Aharoni *et al.* (Aharoni et al., 2002), which explains the metabolic changes during strawberry ripening and to differentiate transgenic and non-transgenic plants, more recent applications are emerging for phenotyping studies (Hirai et al., 2004, 2005; Murch et al., 2004; Brown et al., 2005; Tohge et al., 2005). This technology requires specialized skills and equipment, not easily accessible to most researchers. Also the high per sample cost and the inability to separate structural isomers, which have identical mono-isomeric masses, is still seen as a significant limitation to its application.

Fourier Transform-Infrared spectroscopy (FT-IR)
This is a basic spectroscopic technique and is characterized as fast, high-throughput, non-destructive, and non-selective (Ellis et al., 2007). Since the technique is not as expensive as the other spectrometric and spectroscopic techniques, it is a method of choice for an initial screening (Allwood et al., 2006). Analysis of food products like meat (Ellis et al., 2002, 2005) or milk (Aernouts et al., 2011) seems to be favored by this technique. The major disadvantages related to this method are its poor reproducibility and highly intense detection of water molecules (Allwood et al., 2007).

Nuclear Magnetic Resonance Spectroscopy: Principles and Techniques
In NMR any molecule with one or more atoms with nonzero magnetic moments can be detected, such as ^{1}H, ^{13}C, ^{14}N and ^{31}P. Of these ^{1}H NMR is the most commonly used in metabolomics studies since hydrogen is part of most organic metabolites and because of the high abundance (99.98%) of the ^{1}H isotope (Krishnan et al., 2005). A basic solution ^{1}H NMR experiment consists of placing a liquid sample in a glass tube between two poles of a powerful magnet. The magnetic moments of the protons in the sample, previously randomly aligned, become aligned in the direction of the magnetic field. A radiofrequency signal is transmitted into the sample, which perturbs the magnetization vector of the sample. After the pulse the vector eventually returns to equilibrium, and this change in magnetization over time (called free induction decay, FID) is monitored and from this a signal is evolved. Fourier transformation is used to transform the signal from the time domain to the frequency domain, resulting in a spectrum of intensity

Table 1. Examples of pulse programs and processing parameters for some 1D and 2D NMR experiments.

Experiment	Acquisition parameters	Processing parameters
1D ^{1}H NMR with water presaturation	Pulse sequence comprising [relaxation delay-60°-acquire] where pulse power is set to achieve 60° flip angle, 10 kHz spectral width, and water saturation is applied during 1.5 s relaxation delay.	Zero-fill to 64 k data points, apply exponential line broadening of 0.3 Hz, apply Fourier transformation, manually phase spectrum (zero and first order corrections), manually correct baseline, calibrate chemical shift to internal standard.
J-resolved NMR	J-resolve pulse sequence, two-pulse echo sequence (relaxation delay-90°-[t1/2]-180°-[t1/2]-acquire), water presaturation during 1.5 s relaxation delay. Acquire FID using data matrix 64 x 4096 points covering 66 x 6361 Hz, with 16 scans for each increment.	Zero-fill to 128 x 4096 and apply sine bell-shaped window function in both dimensions before magnitude mode 2D Fourier transformation. Tilt resulting spectra along rows by 45° relative to frequency axis and symmetrize about the central line along F2. Manually correct baseline, calibrate chemical shift to internal standard.
COSY (^{1}H-^{1}H)	Use phase sensitive/magnitude mode standard three pulse sequence with presaturation during relaxation delay of 1s. Acquire FID using data matrix 512 x 4 096 points covering 6 361 x 6 361 Hz, record with 8 scans for each increment.	Zero-fill to 4096 x 4 096 and apply sine bell-shaped window function shifted by /2 in the F1 and /4 in the F2 dimension before Stated-TPPI type 2D Fourier transformation. Manually phase all spectra, correct baseline and calibrate chemical shift to internal standard.
HSQC (^{1}H-^{13}C)	Use a data matrix of 254 x 4 096 points covering 27 164 x 6 361 Hz with *256 scans* for each increment with relaxation delay of 1s.	Qsine (SSB = 2.0) used for window function. Coupling constants optimized to 145 Hz.
HMBC (^{1}H-^{13}C)	Use a data matrix of 254 x 4 096 points covering 27 164 x 6 361 Hz with 256 scans for each increment with relaxation delay of 1s.	Data should be linear predicted to 512 x 4096 points using 32 coefficients before magnitude type 2D Fourier transformation and apply a sine bell-shaped window function shifted by /2 in the F1 and /6 in the F2 dimension. Calibrate chemical shift to internal standard (^{1}H and ^{13}C chemical shift).

versus frequency. Each signal corresponds to a specific proton in a molecule, the frequency or chemical shift (in unit ppm) of which depends on the kinds of protons found in a molecule and how they are arranged relative to each other.

One-Dimensional Nuclear Magnetic Resonance (^{1}H and ^{13}C NMR)

A pulse program for a basic one-dimensional ^{1}H NMR experiment used in metabolomics studies is shown in Table 1. The integrated areas under ^{1}H NMR signals are directly proportional to molar concentrations of the corresponding protons. For very accurate quantitation by ^{1}H NMR, certain pulse program parameters may need to be further optimized (Pauli et al., 2005). All ^{1}H NMR signals can be quantitated with one internal standard. Commonly used internal standards in aqueous solvents are trimethylsilyl propanoic acid (TMSP) and 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS), but others may be more suitable in different NMR solvents (Pauli et al., 2005).

In contrast to ^{1}H NMR, signal intensity in ^{13}C NMR is not always directly proportional to the number of carbon nuclei. This is mainly due to the long and variable relaxation times of different carbon nuclei in different molecules. This, together with the low sensitivity of ^{13}C NMR, makes standard ^{13}C experiments less suitable for accurate quantitation. These problems can be overcome by using inverse-gated ^{1}H decoupling (Freeman et al., 1972). This requires long inter-pulse delays resulting in often prohibitively long acquisition times. Methods have, however been developed to make such measurements faster (Giraudeau and Baguet, 2006).

In many types of samples the presence of residual water causes a large, broad signal in the ^{1}H NMR spectra, which can interfere with other metabolite signals (Gottlieb et al., 1997). Water suppression is commonly included in pulse sequences to overcome this problem. Pre-saturation is often used, where selective pre-irradiation of water resonances equalizes the spin populations of water protons before the data is acquired. This prevents water from contributing to the NMR spectrum. Another commonly used method in metabolomics studies combines pre-saturation with a one-dimensional version of a two-dimensional ^{1}H-^{1}H-NOESY sequence. This and other water suppression methods are discussed thoroughly by McKay (2009) and Price (1999). Some of these methods can also be used to suppress large signals belonging to components other than water. In analysis of alcoholic beverages, for example, the ethanol signal may be very large. The WET technique (water suppression enhanced through T_1 effects) is one way to suppress this dominating signal (Ogg et al., 1994). Kosˇir and Kidric (2000) incorporated a WET pulse sequence element into 1- and 2-D NMR experiments to analyze amino acids in wine, allowing their full signal assignment. A further potential source of interference in ^{1}H NMR spectra is peak broadening caused by macromolecules (e.g. proteins, polysaccharides). In samples where this may occur,

such as biofluids, a modified pulse sequence such as the Carr-Purcell-Meiboom-Gil spin echo modification can be used. This makes acquisition more selective for low molecular weight compounds (Viant, 2007).

In a complex mixture a ^{1}H NMR spectrum is usually a crowded plot with much signal overlap. Such a plot can be used in multivariate data analysis to compare the overall signal patterns between samples. However, metabolite identification and quantification is hampered by overlapping signals, so additional NMR experiments, multidimensional NMR is usually needed. ^{13}C NMR produces 1D spectrum with a wider chemical shift spread and less overlap between signals. Low sensitivity and long acquisition times may prohibit the use of ^{13}C for large metabolomics studies. For certain sample types, however, ^{13}C NMR offers advantages over ^{1}H NMR. For the analysis of vegetable oils (e.g. olive oil) for example ^{13}C NMR provides structure-specific information that cannot be obtained with ^{1}H NMR or even the more traditional GC methods (Sacchi et al., 1998; Vlahov 2006).

J-Resolved Spectroscopy (JRES)

Two-dimensional ^{1}H *J*-resolved (JRES) NMR spectroscopy is a homonuclear method that can be used to generate less congested spectra. Chemical shifts and spin-spin couplings are visualized along two different axes, which make it easier to tell signals apart in crowded regions of the spectra (Viant and Ludwig 2010). An example of a portion of a *J*-resolved spectrum is shown in Figure 1a. The J-resolved spectrum can also be projected on the chemical shift axis to generate a proton-decoupled projected 1-dimensional spectrum (p-JRES) (Viant 2003). In such spectra all ^{1}H signals appear as processed singlets, resulting in much simpler and better resolved signals more suitable for quantitation (Choi et al., 2006). p-JRES spectra can also be used in multivariate data analysis, where simplified spectra may improve interpretability of analyses (Viant 2003). One potential problem to look out for using p-JRES is the presence of "strong coupling artifacts". They occur when strong coupling leads to additional peaks in the J-RES spectrum. When the signals are projected onto the chemical shift axis, the projection does not represent a fully decoupled 1D spectrum, since additional peaks appear. Some methods for suppression of such artifacts were proposed by Thrippleton et al (2005). Even though the acquisition time per sample is about double that of standard ^{1}H NMR measurements, the advantages of using p-JRES spectra may outweigh these drawbacks.

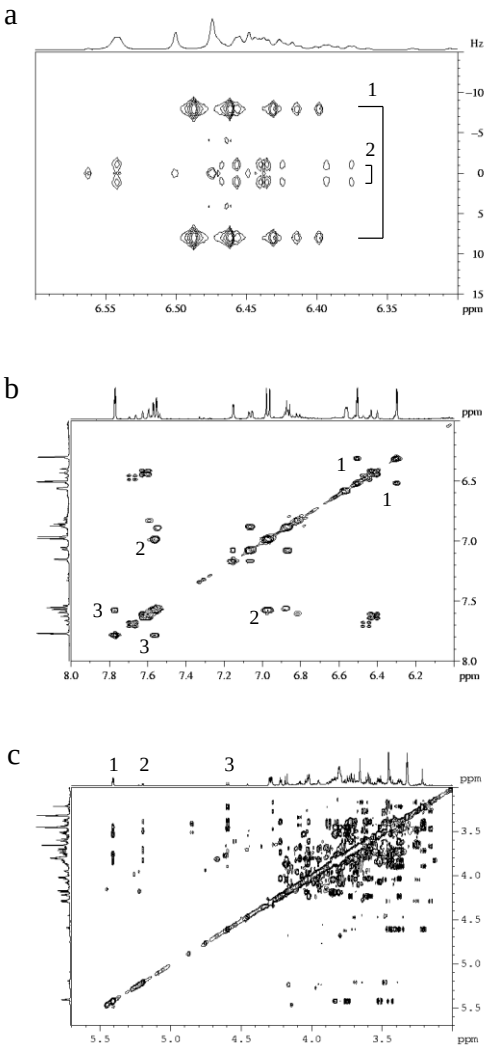

Figure 1. Examples of two-dimensional homonuclear ^{1}H-^{1}H experiments of various plant samples recorded in CH_3OH-d_4-D_2O (KH_2PO_4 buffer, pH 6.0). **a.** ^{1}H-^{1}H-J-resolved spectrum of *Brassica rapa* leaves in the range of δ 6.3 – δ 6.6. 1; H-8 of phenylpropanoids, 2; H-6 or H-8 of 5,7-dihydroxyflavonoids. **b.** COSY spectrum of *Vitis vinifera* leaves in the range of δ 6.6 – δ 8.0. 1; Correlation between H-6 and H-8 of quercetin analogues, 2; Correlation between H-5′ and H-6′ of quercetin analogues, 3;

Correlation between H-1′ and H-6′ of quercetin analogues. **c.** TOCSY spectra of *Cannabis sativa* flowers in the range of δ 3.0-5.7. 1; sucrose, 2; α-glucose, 3; β-glucose.

Correlation Spectroscopy (COSY)

Correlation spectroscopy (COSY) is another homonuclear two-dimensional method that shows correlations between protons with mutual spin-spin couplings. The one-dimensional NMR spectrum runs diagonally across the plot, with spin-spin couplings (J-couplings) indicated as cross-peaks in the off-diagonal space (Xi, Yuanxin et al., 2006). COSY can indicate couplings between multiplets three bonds away, but long range coupling between protons four bonds away may sometimes be seen. In plant samples this method is particularly useful for metabolite identification in the aromatic region of the spectrum (around 6.0-8.0 ppm). This is where many secondary metabolite signals occur, often with a large degree of overlap and at low signal intensity. For example trans-phenylpropanoids in plants typically have doublet signals at 6.3-6.5 ppm (J = 16) with COSY correlations to doublets at 7.3-7.85 ppm (J = 16) (Jahangir et al., 2008). With the help of COSY and J-Resolved spectra, Ali et al. (2009) assigned various phenylpropanoid and flavonoid signals in grapes, including quercetin-3-O-glucoside and a trans-feruloyl derivative associated with resistance to downey mildew. An example of a COSY spectrum is shown in Figure 1b.

Total Correlation Spectroscopy (TOCSY)

While COSY shows correlations between geminal or vicinal protons of a molecule, two dimensional Total Correlation Spectroscopy (TOCSY) creates correlations between all protons in a spin system, as long as there are couplings between every intervening proton. TOCSY allows one to see which signals belong to the same molecule, and is particularly useful for assigning carbohydrate and amino acid signals in the usually crowded region where they occur (Figure 1c). Another variant, selective TOCSY is a 1-D method where a selected signal is excited and the excitation is progressively transferred along all coupled protons in the spin system. This can be done in a complex mixture to give rise to a 1-D spectrum showing only the peak of interest and signals of the same spin system. Overlapping signals can be resolved to help confirm metabolite identities, and to aid in quantitation of minor compounds (Sandusky and Raftery, 2005).

Heteronuclear two-dimensional methods

Heteronuclear two-dimensional NMR experiments such as Heteronuclear Single Quantum Coherence (HSQC), Heteronuclear Multiple Quantum Coherence (HMQC) and Heteronuclear Multiple Bond Coherence (HMBC) are used to show correlations between ^{13}C and ^{1}H. HSQC and HMQC both generate plots of the ^{13}C spectrum versus the ^{1}H spectrum, with direct correlations (J_1) indicated as cross-peaks in the plot (see Figure 2a and b). Both experiments provide the same information, but in HMQC

broadening of resonances by homonuclear coupling can occur. For this reason better resolution can be obtained in the carbon dimension using HSQC. Some examples where this method is useful for signal assignment include anomeric carbons of carbohydrates (δ 90-110), C-6 and C-8 of flavonoids (δ95-110) and methyl groups of terpenoids (δ10-25) (Kim et al., 2006). Similar to p-JRES, an F_1 projected HSQC spectrum can be produced to generate new one-dimensional variables containing additional information from ^{13}C for use in multivariate data analysis (Kim, Saifullah, et al., 2010). HMBC shows long range correlations (J_2, J_3) between ^{13}C and ^{1}H. This is very useful in assigning quaternary carbon signals, as was illustrated for progroitin and other secondary metabolites in *Brassica rapa* leaves (Abdel-Farid et al., 2007). In addition is it also a valuable tool to confirm structures of molecules.

Combined two-dimensional methods

Two-dimensional NMR experiments can be combined to help resolve overlapping signals. An example is the 2D HSQC-TOCSY experiment, where a TOCSY mixing sequence is added after an initial HSQC pulse sequence. This extends the original proton-carbon correlation peak onto neighboring protons within the same spin-system to produce a ^{13}C-dispersed TOCSY spectrum (Figure 2c). Cross peaks in such a spectrum will indicate correlations between all J-coupled protons and all carbons in that spin system. Similar information can be obtained by combining the HMQC and COSY experiments (Hu et al., 2011). A good example of the application of combined 2D experiments for metabolite identification in a complex mixture was described by Leiss et al (2009). HSQC-TOCSY, together with other 2D NMR experiments, was used to find candidate metabolites important for the difference between thrips-resistant and susceptible *Senecio* hybrid plants.

NMR for metabolomics

Nuclear magnetic resonance spectroscopy has been widely applied in plant metabolomics and is the first choice for medical metabonomics (Choi et al., 2004; Defernez et al., 2004; Liang et al., 2006). However, NMR application in metabolomics studies is often criticized because of its poor sensitivity (Kaddurah-Daouk et al., 2004). The sensitivity of NMR is determined among others by the time of accumulation of the spectra. With a standard 500 MHz NMR, a spectrum of an extract of 50 mg dry weight plant material can be obtained in about 10 minutes (128 scans). Higher field strength, cryoprobes, and microprobes have greatly contributed to the shorter time needed to record a spectrum in NMR spectroscopy which makes it competitive with chromatographic methods. Also the amount needed in most type of experiments is not limiting. So sensitivity is more related to the dynamic range and the overlapping of signals.

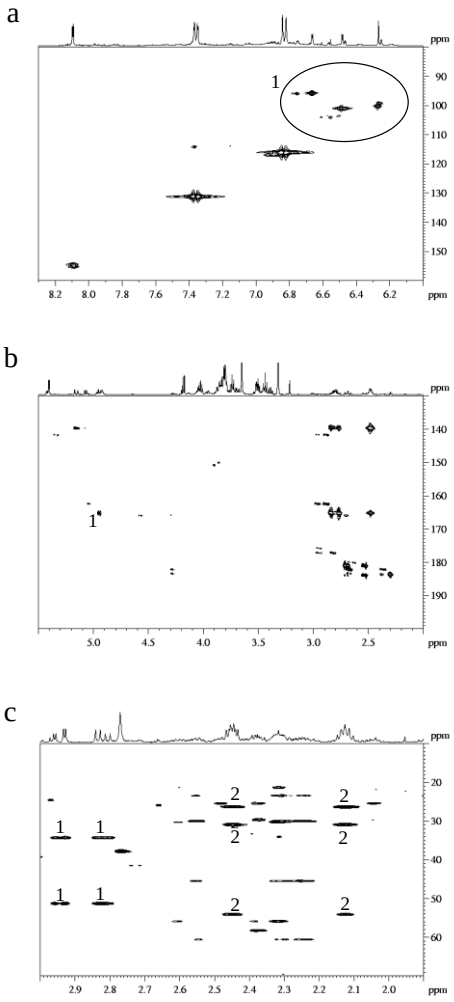

Figure 2. Examples of two-dimensional heteronuclear ^{13}C-^{1}H experiments of various plant samples recorded in CH_3OH-d_4-D_2O (KH_2PO_4 buffer, pH 6.0). **a.** HMQC spectrum of *Genista tenera* leaves in the range of δ 1.9- δ 3.0 (^{1}H) and δ 80- δ160 (^{13}C). 1; C-6 or C-8 of 5,7-dihydroxyflavonoids. **b.** HMBC spectrum of *Brassica rapa* leaves in the range of δ 2.0- δ 5.5 (^{1}H) and δ 130- δ 200 (^{13}C). 1; correlation between anomeric proton and carbon of C=N in glucosinolates. **c.** HSQC-TOCSY spectrum of *Nicotiana*

plumbaginifolia cell lines in the range of δ 6.0- δ8.3 (^{1}H) and δ 10- δ 70 (^{13}C). 1; carbons of aspartic acid, 2; carbons of glutamic acid.

Nuclear magnetic resonance has some unique advantages over chromatography and MS based methods. First, it is a more uniform detection system and can directly be used to identify and quantify metabolites, even *in vivo*. The most promising features of NMR are its non-destructive nature, simple sample preparation in relative short time or even direct measurement of samples e.g. urine. Another major advantage of NMR is that quantification is easy for all compounds. With a single internal standard all the detected metabolites can be quantified without the need of calibration curves for each single compound as signal intensity is only dependent on molar concentration. Since nearly no sample pretreatment is required in NMR spectroscopy, the inherent properties of the sample are well kept. The non selectiveness of NMR makes it an ideal tool for the profiling of a broad range of metabolites (Dixon et al., 2006). NMR is a robust and reproducible method and is much less affected by instrumental and experimental factors as in other analytical methods. This makes it a suitable method for building up databases for long term use. Continuous improvements in instrumentation design may lead to increasing popularity of this approach and a full overview of the current potentials and limitations of NMR has been provided by Ratcliffe and Shachar-Hill (2005) and recently by Schripsema (2010).

Sample preparation

Most NMR-based metabolomics studies are performed with solution NMR. For biofluids (e.g. urine, plasma) minimal processing is needed as samples are already in liquid form (Beckonert et al., 2007). In plant studies, the low molecular weight compounds making up the metabolome needs to be transferred from the plant matrix into a deuterated solvent for analysis by NMR. This sample preparation usually involves the following steps: harvesting, drying, extraction and preparation for analysis (Kim and Verpoorte, 2010). Sample preparation is a very critical part of a metabolomics experiment, as it will determine the quality of the results obtained. For each step in the process a number of practical considerations should be taken into account.

If plant material is to be harvested on different days, it should ideally be done at the same time of day. It is well known that levels of certain plant metabolites fluctuate throughout the day (Queiroz, 1974). The plant part to be harvested should be chosen carefully. If possible organs should be analyzed separately, as large differences in metabolic profiles may be seen between them. Also organs of the same type, but of different ages may differ considerably in metabolite levels. Since plant cells are often very specialized, one should bear in mind that the metabolic profile of for example a leaf is in fact a mixture of many different cell metabolomes.

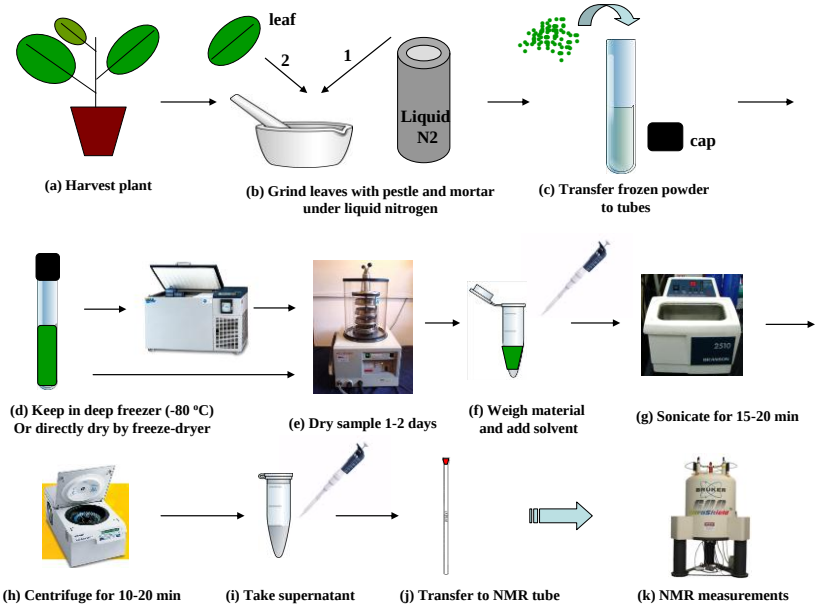

Figure 3: Schematic representation of the experimental procedures for sample preparation (Adapted from Kim *et al.* 2010).

While removing the fresh plant material from the original plant, the aim to is to keep all metabolites in their original state. Handling and wounding can break cell compartments, and unwanted enzymatic and chemical reactions can lead to undesired changes or degradation of metabolites. The best way to avoid this is the rapid freezing of fresh plant material in liquid nitrogen. This stops any enzymatic activity and allows further processing or storage (at -80°C) without any metabolic changes occurring as long as it is frozen. At this point plant material may be ground in liquid nitrogen to homogenize tissue for ease of handling and improved extraction.

In some well-established methods, extraction with perchloric acid ($HClO_4$) proceeds from this point, leading to denaturation and precipitation of unwanted proteins and extraction of polar metabolites (Kruger et al., 2008). An alternative is to first dry the plant material, which helps stabilization by removing the matrix in which enzymatic reactions take place. Removing the water also reduces the interfering water signal in NMR spectra, as well as variable chemical shifts caused by differences in pH. In addition, drying the samples allow for more accurate quantitation of metabolites. Various methods can be used for drying plant material, but freeze-drying is the most commonly used. Except for some volatile compounds, most compounds are well

preserved during freeze-drying, which is mild in comparison to other methods (Kim and Verpoorte, 2010). Typically 50-100 mg of dry plant material is extracted per sample, but less can also be used. For smaller amounts it is very important that the material is homogenously ground. The aim of solvent extraction in sample preparation for metabolomics is to obtain an accurate snap shot of the metabolome. This is not a simple task as many factors like solvent, time, temperature, pH, energy, solubility, and dissolution rate influence the extraction process. The huge diversity in structure, concentration range and polarities of the low molecular weight metabolites in any given tissue also means that no single solvent can extract all low molecular weight metabolites. Many extraction methods have been employed in the past and each one has advantages and disadvantages. The method of Kruger *et al.* (2008) is very good for polar metabolites, but excludes hydrophobic compounds and may also cause degradation of acid-labile metabolites. Organic solvents in mixtures with other organic solvents or water are often used to increase the range of metabolites extracted. A two-phase solvent system comprised of chloroform, methanol and water (2:1:1, v/v) is one such combination. This method produces two extracts per sample, one with more polar and the other with non-polar compounds. More information can be obtained from the analysis of the two phases, and it has been shown to be useful for plant materials where hydrophilic secondary metabolites are causing discrimination between samples (Choi et al., 2004b,c). A drawback of this method is that it requires time-consuming separation and evaporation steps, as well as the need to reconstitute the extracts in NMR solvents. Extraction can be simplified by directly performing it in deuterated NMR solvents. This narrows the choice of solvents somewhat since not all solvents are available in deuterated form. The combination of deuterated water and methanol (1:1, v/v) was found to be a good general purpose solvent for the extraction of a range of primary and secondary metabolites. Replacing the water with a phosphate buffer (pH 6.0) avoids signal shift due to pH variation. This extraction solvent (CH_3OH-d_4-KH_2PO_4 buffer in D_2O, pH 6.0) has now become a well-established method for metabolomics studies on a range of plants (Kim 2010).

Apart from the solubility of compounds in a chosen extraction solvent, the efficiency of an extraction also depends on the dissolution rate. This can be increased by increasing the extraction time, or by adding energy to the system (through heat or using methods such as ultrasonication or microwave extraction). Care should be taken however, as using such methods to improve the extraction yields also increase the risk of artifact formation (Maltese et al., 2009).

Metabolite identification

Even with all the 1- and 2D NMR experiments available, structure elucidation of metabolites can be very challenging and time-consuming, especially in a complex

mixture. The rapid acquisition of many ^{1}H NMR spectra for a metabolomics study is often offset by the long time needed to assign metabolite signals in complex mixtures. Comparing signals to spectral data of reference compounds measured in the same conditions is the most straightforward way of compound identification. Availability of reference compounds may be a problem, especially with secondary plant metabolites. Even with reference spectra manually comparing signals is a time-consuming task.

Statistical methods can be used to aid metabolite identification. One such method is statistical Total Correlation Spectroscopy (STOCSY), which identifies peaks belonging to the same molecule in a mixture by them being highly correlated (Cloarec et al., 2005). Many spectra are analyzed simultaneously and in addition to information about intramolecular connectivity, negative or low correlations between signals can indicate intermolecular connections via a metabolic pathway or common regulatory mechanisms (Couto Alves et al., 2009). This is a useful tool in metabolic studies, however care should be taken in interpretation as results may differ depending on how many spectra are being compared.

Various computational methods have been developed to automatically or semi-automatically identify and quantify metabolites in NMR spectra. The ChenomX NMR Suite is an example of a software package that semi-automatically identifies compounds from ^{1}H NMR spectra (Holmes and Antti, 2002). A spectral library of about 260 compounds is used for the peak fitting process. While this method speeds up the identification process, the manual nature of the fitting and analysis may lead to inconsistent interpretations when used by different individuals. A more automated method developed by Zheng et al. (2011) uses linear mixed modeling with Bayesian model selection on local regions of ^{1}H NMR spectra for metabolite identification and quantitation. This method simultaneously models the entire collection of spectra, and unlike other models performs identification and quantitation at the same time to give improved results compared to similar methods. MetaboMiner was developed to identify metabolites in biofluids based on 2D NMR spectra (Xia et al., 2008). Prior knowledge on biofluid type is used to achieve this semi-automatically with very high accuracy. Unlike some of the aforementioned methods this program does not provide quantitative analysis of the identified metabolites. A method developed by Xi *et al.* (Xi et al., 2006) also used a 2D NMR (COSY) based method to identify metabolites in complex mixtures through comparison with a library of pre-collected spectra. The method gave good accurate results but may have limited practicality since the spectral library contained only 19 recorded spectra.

These studies show it is possible to automatically assign a signal or provide a list of potential assignments. Ideally these methods should be linked to large spectral databases with many entries. Many of the aforementioned methods were linked to small databases containing only a few tens or hundreds of metabolites, which limits its

usefulness. Several large online databases exist with NMR spectra of small molecules (Table 2). In some cases (e.g. HMDB, MMCD, BMRB) they can be queried directly with peak lists from a compound mixture.
Standardization of acquisition conditions is essential for such databases to be useful, particularly for ^{1}H NMR spectra where solvent effects and pH can cause variation in chemical shift. ^{13}C-NMR chemical shifts are less sensitive to small differences in the surrounding environment, and it has therefore been suggested that 2-D ^{13}C-^{1}H spectra (e.g. HSQC) should always be included in spectral databases (Kikuchi and Hirayama, 2006).

Data analysis
Although different in many ways, all the platforms for metabolomics studies produce large amounts of data. In order to generate information, and eventually knowledge, this raw data need considerable processing and then suitable statistical analysis. The success of any metabolomics based study relies on how efficiently the data is processed and analyzed. Since metabolomics data can rarely be handled manually, the processing and analysis require specialized bioinformatics tools for the *in silico* multivariate analysis of this data set. A brief account of pre-processing of NMR data is presented here, followed by some multivariate data analysis methods used in NMR-based metabolomics.

Data pre-processing
In NMR-based metabolomics studies, the spectroscopic data needs to be processed before proceeding with the multivariate data analysis. Several methods to process the NMR data, like peak alignment, normalization, scaling and bucketing (binning) are available and explained briefly in this chapter.
Resonances of the same metabolite in the NMR spectra of different samples (e.g. replicates) can sometimes be shifted to have slightly different chemical shifts. This usually happens because of instrument variations or differences in the sample properties such as pH. These shifts can be corrected in the process called peak alignment. Another type of variation is in the concentrations of the metabolites. This difference in concentration hinders the quantification (absolute or relative) of metabolites and is addressed by a process of normalization. For normalizing the signals, a stable standard of known concentration is added to the sample and the rest of the spectrum normalizes with respect to that internal standard. The signal of NMR solvent can also be used for normalization. This data pre-processing is essential for the correct quantification of the identified metabolites.
If large metabolic differences existed among the analyzed samples, scaling is needed to reduce the influence of highly inconsistent signals (or variables). There are many ways to scale the data before proceeding with the multivariate data analysis. The most basic is

mean centering, which is done with each variable across all the samples. In mean centering, the mean value for each variable is calculated and then subtracted from the data. Another widely applied scaling method is unit variance (UV) in which each variable is divided by its standard deviation to give each variable an equal variance. This method of scaling is recommended if no prior information of the data is available.

Sometimes 'no scaling' is appropriate, especially if the data is in the same unit, e.g. spectroscopic data. Another development in scaling methods is Pareto scaling in which each variable is divided by the root of its standard deviation. Pareto scaling scales each variable according to its initial standard deviation and is intermediate between no scaling and UV scaling.

Bucketing or binning is another type of data processing, which divides the NMR spectrum into a number of discrete spectral regions of desired width and calculates the peak areas within that segment of the spectrum. Bucketing can help to reduce the effects of pH-induced variations in the chemical shifts by ensuring the measurement of the same resonance across the samples. Since bucketing calculates the peak areas, it is also helpful to quantify the metabolites, as the peak intensities in NMR spectrum are proportional to the molar concentrations of the metabolites. A bucket width of 0.04 ppm is commonly used but this can vary depending on the application.

Principal component analysis (PCA)

Principal component analysis (PCA) is the most common multivariate data analysis method, which is used to reduce the dimensionality of a multivariate dataset by data decomposition. This method is designed to extract the maximum variation from the X-data matrix. Since this modeling is done solely on the explanatory variables and without any prior information of samples, PCA is an unsupervised and hence unbiased method. This method generates score and loading vectors and can be represented in a graphical form known as a score plot and loadings plot, respectively. The score plot can be use to identify the differences or similarities among the samples with easy identification of an outlier. The loadings plot can be used to identify the spectral signals responsible for the grouping or separation among the samples which ultimately resulted in the identification of metabolites responsible for the separation on the score plot.

Table 2. Online Databases for NMR spectroscopy

Spectra	Name	URL	Developed/maintained by
NMR spectra of proteins, peptides, nucleic acids	Biological Magnetic Resonance Data Bank (BMRB)	www.bmrb.wisc.edu	University of Wisconsin-Madison
More than 48 000 measured NMR spectra (^{13}C, ^{1}H, ^{15}N, ^{11}B, ^{19}F, ^{29}S, ^{31}P)	NMRshiftDB	http://nmrshiftdb.org	University of Mainz, Max Planck Institute Chemical Ecology in Jena, European Bioinformatics Institute in Cambridge
More than 20 000 small molecules, ^{1}H and ^{13}C spectra, 1D and 2D.	Madison Metabolomics Consortium Database (MMCD)	http://mmcd.nmrfam.wisc.edu/	University of Wisconsin-Madison
More than 7900 human metabolite NMR spectra (^{13}C and ^{1}H)	Human Metabolome Database (HMDB)	www.hmdb.ca	University of Alberta.
More than 27 000 NMR spectra (^{13}C and ^{1}H)	Spectral Database for Organic Compounds (SDBS)	http://riodb01.ibase.aist.go.jp/sdbs/cgi-bin/cre_index.cgi	Japanese National Institute of Advanced Industrial Science and Technology
NMR spectra of standard compounds, also other tools for signal assignment and integration of –omics data	PRIMe	http://prime.psc.riken.jp/	RIKEN Plant Science Center, Yokohama
NMR spectra of mostly primary, and some secondary metabolites	NMR metabolomics database of Linkoping	http://www.liu.se/hu/mdl/main/	University of Linköping, Sweden
NMR spectral matching with databases and predictor tools (^{13}C, ^{1}H, ^{15}N, ^{31}P)	Advanced Chemistry Development	www.acdlabs.com	ACDLabs
NMR spectral databases (^{13}C, ^{1}H, ^{19}F, ^{31}P, ^{29}Si)	SpecInfo on the Internet	http://onlinelibrary.wiley.com/book/10.1002/9780471692294	John Wiley & Sons
^{13}C and ^{1}H spectra of more than 11 800 metabolites	The Aldrich FT-NMR Library	http://www.sigmaaldrich.com/analytical-chromatography/spectroscopy/learning-center/nmr-spectroscopy/spectral-viewer.html	Sigma-Aldrich

Partial least squares projections to latent structures (PLS)

As a regression extension of PCA, PLS based modeling is the basis of supervised multivariate data analysis algorithms. When sample specific information is available as a Y-data matrix (bioactivity data, for instance), this method often does more efficient data decomposition than PCA and can connect the X-matrix (descriptor) and Y-matrix (response) to each other. Similar to PCA, score and loadings plots are used to visualize the respective score and loadings vectors. Another PLS-based technique, known as partial least squares-discriminant analysis (PLS-DA), is capable of separating 'tight' (with least internal variation) classes of observations based on their X-data matrix. In PLS-DA, the Y-matrix represents class membership by a set of 'dummy' variables. This method separates the observations according to the class membership and a discriminant plane is found by fitting a PLS-model between and X and artificial Y matrices. A low number of classes (not more than 5 or 6) and high degree of tightness are required for this model to work efficiently.

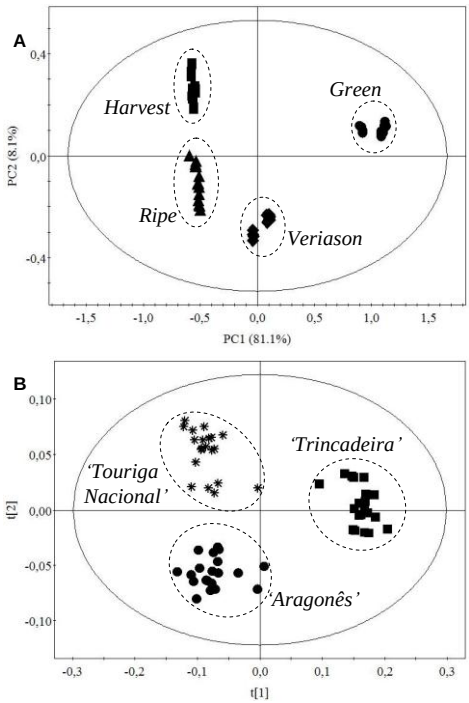

Figure 4: Score plots of different multivariate data analysis. Score plot of PCA (A) shows the clustering of samples according to the developmental stages of grape berries. Score plot of PLS-DA (B) shows the classification of grape berries based on the cultivars (Adapted from Ali *et al.* (2009)).

Bidirectional orthogonal-PLS (O2PLS)

Methods like PLS regression can cause systematic variation due to structured noise present in the data matrices. Other algorithms, like O2PLS, is an extension of PLS regression with an integrated OSC (orthogonal signal correction) filter. The OSC filter, initially developed to pre-process spectral data by Wold et al. (1998), can identify the systematic variation in the X-matrix by employing information regarding the Y-matrix. Depending on the study, this information can be removed or retained. Similarly analyses like O2PLS (and O2PLS-DA for discriminant analysis) are multivariate projection methods which remove the structured noise by extracting linear relationships from independent and dependent data blocks, in a bidirectional way, and results in the decomposition of systematic variation into two model parts: the predictive or parallel part and the orthogonal part (Trygg and Wold, 2002; Trygg and World, 2003). These methods are really effective in correlating two data types (spectral and activity data, for instance) or for the integration of different omics as illustrated in the applications section of this chapter.

Validation

The methods described above are very useful but they are sensitive to false-correlation or over-fitting. Conclusions on the basis of false-correlated or over-fitted models can be disastrous, especially in the case of medical based studies. Different validation tools, like cross-validation, permutation tests, Jack-knifing model parameters, and test-set validation, are available to overcome this problem (Rubingh et al., 2006). The three most widely applied validation methods in metabolomics are cross-validation, permutation tests, and test-set validation.

Cross-validation is a technique which divides data into equally-sized blocks. The idea is to use the available data blocks minus some blocks to fit the model and the left out data blocks to test the model. Since the fitting and testing of the model is done on the same original data, this method is biased and to further test the model a separate data set is required. This problem can be overcome by using double cross-validation. The significance of classification can also be evaluated by a permutation test. In permutation testing, permutation of the class assignment is done several times and a model between the data and the class assignment is built for each permutation. Also the discrimination of the model based on original classification is compared with the discrimination between the classes of the model based on the permutated-class assignment. The test-set validation involves a separate data set (treated in the same way as the training data) to check the validity and fitness of the statistical analysis. If the test data is kept separated, the reproducibility and accuracy of the produced model will be discovered by

validation. It is also possible to divide a large data set into bigger training data set and smaller test data set, in case a separate test data set is not available.

Applications of NMR-based metabolomics

Metabolite profiling or fingerprinting is being used in a broad range of fields. It has become a useful approach in fundamental scientific work as well as more applied research. Some examples of the application of NMR-based metabolomics to plant sciences are discussed in the following sections.

Understanding stress response and plant resistance

Metabolomics is increasingly being used as a platform to understand cellular phenotypes and to study their response under various types of biotic stresses. Choi et al. (2004c) employed an NMR-based approach to characterize phytoplasma infected *Catharanthus roseus* and reported the association of phenylpropanoids and terpenoid indole alkaloids with the plant response towards the infection. Tobacco mosaic virus (TMV) infection to tobacco plants has also been studies by the same group (Choi et al., 2006). This study revealed that upon infection, the host triggered programmed cell death to restrict the spreading of infection. This was followed by the synthesis of signaling molecules, like salicylic acid, to initiate systemic acquired resistance in the host. Many metabolites known to be involved in plant resistance, such as caffeoyl quinic acid, sesquiterpenoids, and diterpenoids, were also elicited in the infected plant.

More recent work on tomato infection using NMR in combination with PCA and PLS-DA has been published by Lopez-Gresa et al. (2010). The report showed the response generated by the tomato plants upon infection with a viroid (citrus exocortis viroid, CEVd) and a bacterium (*Pseudomonas syringae* pv. *tomato*). It was observed that the host responds differently against different type of pathogens. In the case of bacterial infection, phenylpropanoids and flavonoids (rutin) were the main inducible metabolites, while glycosylated gentisic acid was found critical in viroid infection. The NMR-PCA combination was also effective in studying the constitutive host plant resistance in tomato against thrips (*Frankliniella occidentalis*). Resistant tomato species were characterized by elevated levels of acylsugars, which are well known for their negative effect on herbivores (Mirnezhad et al., 2010).

Studies on *Brassica rapa* interaction with bacterial (Jahangir et al., 2008) and fungal (Abdel-Farid et al., 2009) pathogens also revealed the underlying mechanism of this plant to deal with biotic stress. Metabolites like phenylpropanoids conjugated with malic acid and flavonoids were found in higher levels after infection. NMR-based metabolomics was recently applied to grapevine in order to characterize the metabolic

response against Esca disease suggesting the increased production of phenolics with reduction in carbohydrates (Lima et al., 2010).

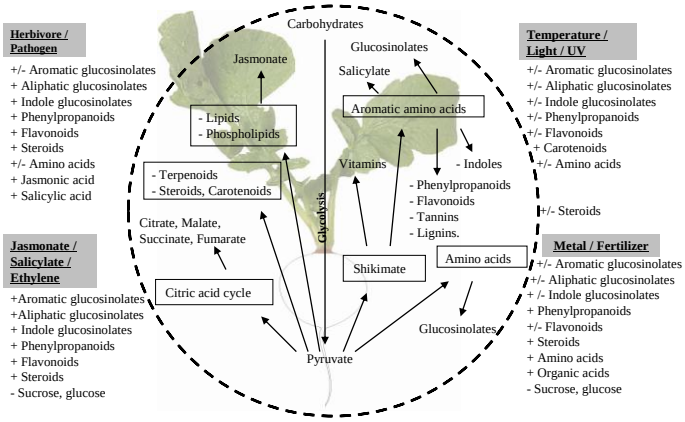

Figure 5: Biosynthetic pathways for the stress-induced metabolites. Different types of stresses are shown by the boxes outside the main circle with the induced metabolites (Adapted from Jahangir et al. 2009).

Application to bioactivity screening

Many plants contain metabolites that have an effect on other organisms. It is often challenging to identify which of the thousands of compounds in a plant is responsible for a given effect (e.g. medicinal or toxic effect on humans, allelopathic effect on other plants). Synergistic effects are also known to occur, where a mixture of several metabolites together have a stronger bioactivity than the expected sum of the individual components. There are several examples where NMR analysis combined with multivariate data analysis was used to correlate particular metabolites to an observed bioactivity.

Roos et al. (2004) evaluated 24 different extracts of four different accessions of St. John's Wort (*Hypericum perforatum*) extracted with six solvents. Partial least squares analysis was used as a regression model and proved effective in identifying the resonances in the ^{1}H NMR spectrum correlated with the pharmacological activity under study. The same NMR-PLS-based approach was used to predict the anti-plasmodial activity in different *Artemisia annua* extracts and allowed their classification based on this activity (Bailey et al., 2004).

A similar approach was used to study the Mexican anxiolytic and sedative plant, *Galphimia glauca*. NMR metabolomics was used to characterize the chemical profile of this plant collected from six different locations along with the discrimination of active and non-active samples using PLS-DA modeling. The signals related to activity were found associated with a specific class of metabolites known as galphimines (Cardoso-Taketa et al., 2008). More recently Cho et al. (2009) successfully reported the use of NMR and PLS modeling for the classification and prediction of free-radical scavenging activity in the fruit of *Citrus grandis* plants at different stages of its development.

Comprehensive extraction of *Orthosiphon stamineus* was combined with NMR-based metabolomics for the screening of Adenosine A1 receptor binding compounds (Yuliana et al., 2011). The method used PLS and OPLS-based modeling to correlate the metabolomics and bio-activity data. In another study TNFα inhibition activity was determined in three grape cultivars at different stages of their development from 2007 and 2008 vintages. NMR spectroscopy and chemometric methods (PLS and O2PLS) were used to identify the active ingredients responsible for the activity. Several phenylpropanoids and flavonoids, which were previously reported to have antioxidant and anti-inflammatory activity, were found positively correlated with anti-TNFα activity. Similar observations were made when different red wines were assessed for the same activity and the proposed methodology was found effective in terms of correlating the activity and metabolomics data and also to identify the active ingredients in the crude wine extracts (Ali et al., 2012).

Using the NMR and chemometrics-based approach, the analysis of NMR shifts in relation to pharmacological activity can provide an idea about what part of the NMR spectrum correlates with the activity and gives information about the active ingredients in crude extracts of medicinal plants. Compounds related to activity can be identified without extensive and elaborate chromatographic separation. This method should be considered as preliminary and final conclusions on metabolite-activity relationship should be drawn on the basis of activity data on the pure compound.

Quality control of herbal medicines

Quality control of herbal medicinal material is an increasingly important issue all over the world. Regulatory authorities demand better standardization of material in terms of active components, and more objective and reproducible analytical methods to achieve this (Wang et al., 2004). The quantitative nature of NMR analysis makes it a very suitable method for accurate analysis of active substances in medicinal plant material. Choi et al. (2003) developed a simple, rapid ^{1}H NMR method for the quantitation of the active compounds in *Ginkgo biloba* leaves and commercial herbal products. Quantitation was performed using the H-12 signal of bilobalide and ginkgolide A, B and C, while separation of flavonoid and other terpenoid signals was also achieved.

Herbal medicines are usually consumed in a crude form (crude extract or powder) so it is often not clear which compounds are responsible for the observed biological activities. Not only does it make quality control more difficult, but variable chemical composition hampers studies into the pharmacological efficacy of the material. A good example is ginseng, which is well known for its adaptogenic properties. The roots of the plant contain compounds of many chemical classes, such as triterpene saponins and many others. In a study by Yang et al. (2006) three commercial ginseng preparations and four kinds of ginseng root were analyzed by NMR-based methods (^{1}H NMR and J-resolved) together with multivariate data analysis. The different samples were clearly discriminated based on the content of various primary and secondary metabolites. This study showed the feasibility of using NMR as a quick method to characterize the overall chemical profiles of ginseng root material.

The ability to analyze many diverse compound types simultaneously makes NMR a suitable method to address purity, another quality aspect of medicinal plant material. It is often difficult to assess the purity of the material in terms of the desired plant part, or assess adulteration with other plant species. Wang et al. (2004) analyzed chamomile flower head samples containing different proportions of stalks by ^{1}H NMR. Principal component analysis classified samples based on content of stalk material, with a linear relationship between the average scores and percentages of stalks. NMR-based metabolic analysis can be used for discriminating medicinal plant species of the same genus. This was illustrated well in a study on *Ephedra* species by Kim et al. (2003). Of the more than 50 species, *E. sinica* is the main species used in medicine. The presence of different species may affect the efficacy of the medicine, as species vary in their active alkaloid profiles. Three *Ephedra* species were analyzed by NMR, and after principal component analysis was shown to be discriminated by alkaloid as well as benzoic acid derivative signals. Further analysis of commercially available *Ephedra* products revealed that most were composed of *E. sinica*, while some were composed of mixtures of species. In a study by van der Kooy et al. (2008) ^{1}H NMR and multivariate data analysis was used to characterize a commercially available herbal antimalarial remedy. Capsules were claimed to contain leaves of *Artemisia afra*, and contain the antimalarial compound artemisinin. Artemisinin has not been reported in *A. afra*, but is well known to be present in the related species *A. annua*. Analysis of leaves of *A. afra* and *A. annua*, as well as the capsules showed a clear differentiation between the two species, with artemisinin an important marker for the discrimination. The herbal capsules were clearly grouped with the *A. afra* samples, and further targeted analysis confirmed the absence of artemisinin.

Chemotaxonomy

The ability to distinguish plant species chemically is not only useful for quality control of medicinal plants, but has also led to the use of NMR-based metabolic analysis in chemotaxonomic applications. A study on 11 species of *Ilex* grown from seed under the same conditions revealed four groups of similar chemical profiles (Kim et al., 2010). PCA and PLS analysis grouped the samples according to their NMR profiles, while hierarchical cluster analysis was applied to these data to show the closeness between species and groups. The results corresponded well with a phylogenetic study of *Ilex* species using DNA-fingerprinting (amplified fragment length polymorphisms).

Safer et al. (2011) used an NMR metabolomics approach together with LC-MS analysis to study relationships between some members of genus *Leontopodium*. Apart from *L. alpinum* (Alpine Edelweiss) not much is known about the other species, especially those occurring in Asia. Twenty three species were analyzed and with the help of PCA and PLS-DA, an unidentified species was shown to be closely related to two others, suggesting that hybridization occurred between them. With the help of DNA-fingerprinting two other species were unambiguously classified, which helped to clear up confusion caused by their very similar morphological characteristics.

Agricultural applications

While chemotaxonomic studies often keep environmental conditions constant to assess the effects of genetics on plant metabolite profiles, other studies grow the same planting stock in different conditions to see how these affect metabolite profiles. Lubbe et al. (2011) conducted a field experiment with *Narcissus pseudonarcissus* cultivated as a medicinal crop to determine the effect of fertilizers on the levels of galanthamine in the bulbs. Quantitative ^{1}H NMR analysis showed that application of different levels of fertilizers changed galanthamine concentration as compared to a control. PCA highlighted differences in metabolite patterns that helped to explain the observed changes in galanthamine in terms of biosynthetic precursors and other metabolites. A study on mandarin oranges in California assessed the effects of various growth conditions on metabolic profiles by ^{1}H NMR and multivariate data analysis (Zhang et al., 2011). Factors like rootstock, soil composition and elevation were found to influence the nutrient composition, which in turn also influences taste profiles of the fruit. The authors concluded that such NMR-based analysis could be very useful in the development of agricultural practices to obtain mandarins with optimized tastes.

Another area of agricultural research to which NMR-based metabolomics has been applied is the study of genetically modified (GM) crops. New genes may be inserted into crop plants to for example improve resistance to pesticides or environmental stress conditions. Alternatively the aim of genetic modification may be to alter the content of specific metabolites related to nutritional or other quality aspects. In the first case, the

new trait should be present without other unexpected changes in the metabolite profile. In the second case the desired effect is to see only a change in the targeted chemical components, without unexpected pleiotropic effects. The ability of NMR-based methods to detect a wide range of chemical compounds together with the unbiased pattern-recognition abilities of multivariate data analysis, make NMR metabolomics useful for seeing unexpected metabolic changes. Some examples of this application in the literature include studies comparing wild-type and transgenic maize (Manetti et al., 2006), tomato (Le Gall et al., 2003), tobacco (Choi, et al., 2004a) and wheat (Baker, et al., 2006). These studies show the potential of such techniques for assessing the effects of genetic modification of plants, but for a more complete understanding of the consequences results should ideally be integrated with other complementary techniques (Rischer and Oksman-Caldentey, 2006).

Future prospects and conclusions

NMR is a powerful analytical tool for the characterization of complex metabolite mixtures. It has been applied to many different sample types, and has found use in diverse applications. From the increasing amounts of publications in the field, it seems that NMR-based methods will continue to make important contributions to the field of metabolomics. Complementing NMR-based methods with other platforms such as MS-based techniques will vastly improve the amount of information obtained. Integrating metabolite data with that of the gene, transcript and protein level also had great potential to increase understanding of organisms as a whole.

The technical strong points of NMR are accurate quantitation and good reproducibility. In contrast sensitivity and resolution remain major challenges in metabolite mixture analysis. The development of stronger magnets is one way to improve sensitivity. Sensitivity can also be improved by the use of cryoprobes, where the NMR probe electronics is cooled to reduce thermal noise. Micro-coil probes allow measurement of small amounts of samples, down to micro or nanoliter volumes. Signal-to-noise ratio can further be improved with the use of microcoils with solenoidal instead of Helmholz geometry (Zhang et al., 2010). Apart from technical advances, experimental techniques may be used to enhance sensitivity. *In vivo* isotope labeling is one such strategy. Cells can be incubated on labeled medium, and plants or invertebrate animals can be fed labeled nutrients so that samples enriched in ^{13}C or ^{15}N can be obtained (Lundberg and Lundquist, 2004; Chikayama, et al., 2008). Signals of these normally low abundance nuclei can be enhanced for more rapid analysis by 2D NMR methods.

Even with more sensitive NMR measurements, signal overlap remains an obstacle to data analysis. As described in this chapter, 2D NMR experiments can help resolve signals allowing easier identification and quantitation. More methods to shorten acquisition times for 2D experiments (e.g. ASAP HMQC,(Kupce and Freeman 2007))

will be valuable for metabolomics studies with great sample numbers. Further development and improvement of automated computational tools for signal deconvolution and assignment may also help speed up the process of data analysis. Availability of large metabolite databases with 1- and 2-D NMR spectra to freely access and link to computational tools will contribute to advances in the field. A number of large databases exist, but there is still a need for more comprehensive plant metabolite libraries.

To improve reproducibility of metabolomics results and facilitate data exchange between working groups, the Metabolomics Standards Initiative was developed (Fiehnet al., 2007). This is coordinated by the Metabolomics Society (www.metabolomicssociety.org/mstandards.html), and is an ongoing project to develop guidelines for reporting of results and meta-data of experiments. Initially the focus was on mass spectrometry as analytical platform, but guidelines for NMR-based studies have also been incorporated (Sumner et al., 2007).

Chapter 3. *Narcissus*: from ornamental to medicinal crop.

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Abstract

Daffodils (*Narcissus* spp.) are well known ornamental flower crops. They have been cultivated for their yellow trumpet-like flowers for centuries, particularly in the Netherlands where cultivation practices are well established. As other members of the Amaryllidaceae plant family, *Narcissus* plants produce various bioactive alkaloids. One of these alkaloids, galanthamine, has been developed and licensed as a drug against symptoms of Alzheimer's disease. An increasing demand for the drug and economic and sustainability issues related to its supply creates the need for alternative sources of galanthamine. This created an opportunity for *Narcissus* growers in the Netherlands to convert their ornamental crop to a medicinal crop. Studies were initiated to assess the feasibility of producing *Narcissus* bulbs as raw material for galanthamine extraction. In order to produce raw material for pharmaceutical extraction, it is important to know which quality standards exist and what is expected from the buyer of such material. This chapter provides an introduction to Good Agricultural Practice for the cultivation of medicinal plant material. The alkaloid galanthamine is introduced, and an overview is given of how *Narcissus* cultivation is typically done for ornamental purposes. This provides a background for the following chapters in which key points critical to the production of high quality raw material for galanthamine extraction will be investigated.

Good Agricultural Practices for cultivation of medicinal plant material

In recent times awareness of and concerns over the environment and the sustainability of agricultural methods have increased. Globalization and international trade has also brought producers and buyers that have vastly different agricultural practices and standards into contact. Good Manufacturing Practices (GMP) for processed goods (e.g. processed foods, pharmaceuticals) have been developed and have become routine parts of business protocols and national and international government policy regulations. This has led to various agencies and organizations (e.g. FAO, WHO) developing recommendations for producing raw materials for use in GMP processes that will be of an appropriate standard and quality. The application of Good Agricultural Practices (GAP) was thus developed as a way to ensure the safety and quality of the whole chain, from production of raw material to final product.

Good Agricultural Practices are a collection of principles to apply for on-farm production and post-harvesting processes, resulting in safe and healthy food and non-food agricultural products, while taking into account economic, social and environmental sustainability. The GAP process embraces actions, technologies and systems that are accepted as the most effective for optimal management of soil and water, and for crop production, from the point of view of microbiological and chemical safety, together with environmental, economic and social sustainability. Details of a GAP protocol for a commodity in a given production environment cannot be generalized, but must be based on general underlying principles and norms while adapted for the local situation. Common principles of GAP include describing farming that uses available technology optimally to promote agricultural productivity of safe and healthy crops, to achieve economic viability and agricultural, environmental sustainability and social responsibility. As described in chapter 1, GAP recommendations were initially set up for food crops, but additional recommendations have been formulated for medicinal plant crops.

The scope of a given GAP scheme should reflect its intended objectives, which can include some or all of the following: ensuring safety and quality of produce and allowing traceability, minimizing environmental impact and creating product differentiation (e.g. in order to capture new market niches). Cultivation systems are not yet in place for many medicinal plants, and if new ones are to be developed it can be done according to specific aims following these guidelines. The following section will try to give a brief overview of the type of information that may be required so that such a production system can be set up in practice. A general overview is given for producing raw plant material for medicinal or pharmaceutical use. Thereafter a specific example, namely the cultivation of *Narcissus* plants (daffodils) as a source of the

alkaloid galanthamine will be discussed in detail.

Quality assurance
An important aspect at the beginning of the GAP process should be defining what is meant by quality. An agreement on what is expected by buyers of medicinal plants/herbal substances from the producers should be laid down in writing, and should comply with recognized regional and/or national specifications. This would be regarding content of active constituents, limit values of chemical residues, heavy metals and microbial contamination. In cases where a whole plant will be used it can be challenging to set standards for active compounds, as often it is not precisely known which of the many metabolites are responsible for the medicinal effect. If one compound is to be extracted from the plant material this may be easier to determine and monitor.

Documentation
An important aspect of GAP is traceability. All processes and procedures carried out during cultivation that could affect the quality of the product should be documented. This may be used in audits to ensure the buyer that the terms agreed on before were adhered to. GAP protocols require documentation to be kept for a certain minimum number of years.

Selection of medicinal plants and botanical identity
Good Agricultural Practice recommendations state that the plant species or variety to be grown should be chosen according to the country's pharmacopoeia, or what is specified in the end-users country's equivalent authoritative documentation. This is assuming that it is a well-known plant with monographs prepared on its medicinal use. If not, research should be carried out to determine the species, cultivar or variety with the most suitable make-up, in terms of chemical constituents and disease resistance. It is very important that the botanical identity of the cultivated plant should be verified. The scientific and common names should be recorded, as well as other relevant information such as chemotype, phenotype and cultivar. All origins of seeds, plants or propagation material should be well recorded. If the plant of interest is not well known, or if there is doubt about its identity, voucher specimens should be submitted to a herbarium for identification. Comparing the genetic pattern of the specimens to an authentic one can confirm identity.

Seeds and propagation materials
It is important that the plant material produced can be traced to its source. The origin of seeds and propagation material should be specified. Commercial suppliers of materials

should as far as possible provide information of the identity, quality and performance of the product, as well as breeding history where relevant. Seeds and materials should be as free as possible from contamination and diseases for healthy plant growth. Any treatment of seeds (e.g. with agrochemicals) should be documented. The quality of planting or propagation materials should comply with regional or national regulations, and all necessary labels and documentation must be present.

Cultivation

If no cultivation method has been established, traditional methods of growing the plant can be followed. Otherwise research should be conducted to develop appropriate methods. Information about the effects of water, nutrients, light and temperature on the secondary metabolite composition of the plants will be needed. Also studies to determine the best planting arrangement in the field will be needed. The presence of weeds in the field should be documented. Weeds can potentially affect the growth of the crop, and can potentially be a source of toxic compounds (e.g. pyrrolizidine alkaloids) when accidentally harvested together with the bulbs. The effect of stress factors (e.g. salt, water, and drought) that may occur should also be studied to see how it would affect the secondary metabolism of the plant. During cultivation any extraordinary conditions or circumstances during growth of the plants should be documented, as it may affect metabolite composition of the plant. Further details of cultivation will depend on whether organic or conventional methods are followed, and on the quality of the product required. Principles of good husbandry should always be applied throughout cultivation, for example the appropriate rotation of crops, and adoption of soil tillage to plant growth and requirements. At all times it should be kept in mind that the environmental impact should be minimized.

Soil

The soil on which plants are grown can have a large effect on the growth and metabolism of the plants. The site chosen to grow medicinal plant crops should contain the appropriate amounts of nutrients, organic matter and other elements for the specific plant. If optimum conditions are not known, trials can be performed to see how soil conditions influence growth and quality. All soil parameters should be documented (e.g. pH, water content, nutrient content, intensity of sunlight, temperature). Additional information about the history of the site should be recorded, such as previous crops grown there, chemicals used before and any contamination with diseases or pests. Fertilizers should be used sparingly, and careful research should be carried out to determine the correct types and quantities needed. Keeping in mind environmental impacts, fertilizers should be applied in such a way as to reduce leaching from the site of use. If manure is used to fertilize the soil, it should be properly composted, and no

human feces may be used. Further soil parameters to take into account are microbial populations in the soil. A healthy mycorrhizal population may have a great impact on health and quality of the plants grown. Monitoring and maintaining microbial populations may therefore be a part of the protocols of the production chain.

Irrigation

Irrigation should be controlled and carried out according to needs of the particular medicinal plant at various growth stages. It is important that the water used should meet national quality standards, for example be free of heavy metals and microbial contamination.

Crop protection

As a rule agrochemicals to promote growth or protect plants should be kept to a minimum. Integrated pest management (the integration of a combination of all available pest control techniques to discourage pest populations and minimize pesticide use and risk to the environment) should be followed where appropriate. Herbicides and pesticides used should be in accordance with recommendations of manufacturers and authorities, not only in the grower's country but also in that of the end-user. Levels of agrochemical residues in the plant material should be checked and reported. If methods to do so are not available they should be developed. Maximum pesticide and herbicide residue limits as described by regional or national regulatory authorities in grower and buyer's countries should be adhered to. Only qualified staff should be allowed to apply agrochemicals, and all applications must be documented. Applications should be in accordance to manufacturers' instructions, and should be carried out with the agreement of the buyer. There have been reports of pesticides and herbicides affecting the metabolism of plants (Deng 2005, Garcia et al 2001, 2003), and this can have consequences for the quality of a medicinal product. If such chemicals are used, studies can be conducted to check the effect of these on the target compound(s) on the metabolome of the plant.

It should be known which pests and diseases are most likely to affect plants in a cultivation system. This will allow the more directed and precise use of agrochemicals. A particular pest may have a significant effect on plant quality. For example attack by certain insects or pests may result in elicitation of defense compounds, which may be desired in higher amounts in the plant material. Attack or infection by other pathogens or pests may cause the presence of unwanted metabolites in the plant material (e.g. mycotoxins, plant defense compounds).

The use of herbicides should be controlled and appropriate ones used according to the

plants that are most likely to grow as weeds with crops. Application should be done carefully to minimize impact of herbicides on non-target plants. Control of weeds are especially important where medicinal plants are grown to be used in crude form, as adulteration and the accidental inclusion of undesired plants can more easily occur if weeds are present at harvest.

Harvest

Time of harvest is very important for medicinal plants as it is well known that concentrations of active compounds vary during growth stages. The optimal time (time of day/year) will depend on the particular species. Information about this can be found in pharmacopoeia, published standards and monographs, or if not available research should be conducted to determine it. Harvest time is not only important for desired compounds in the plant, but also for the presence of potentially toxic and otherwise unwanted non-target compounds. As mentioned before care should be taken when harvesting not to include any foreign substances or unwanted plants or weeds.
As in all other steps in the GAP process, all important parameters related to the harvesting should be documented. These include date and time, and also the weather conditions of harvesting. Harvesting should be avoided on days where conditions are favorable for molds or microbial fermentation to occur, for example on very humid or wet days. Care should be taken upon harvesting to avoid mechanical damage of the plant material. Damage caused by compacting or overfilling of boxes for example can have an undesired effect on the quality of plant material.

Primary processing

After harvesting plant material should be inspected for possible substandard material and for the presence of foreign substances. Depending on the plant material, some primary processing might need to be carried out such as washing, drying, cutting, fumigation, freezing etc. Specific processing may need to be done to improve the purity of the plant part being used, reduce drying time, to prevent mold damage, detoxify toxic ingredients and enhance therapeutic efficacy. Examples of such processes include: peeling skins of roots and rhizomes, steaming, soaking, pickling, distillation, fumigation, roasting, treatment with lime, chopping, and natural fermentation. Any processing performed should be documented and possible effects this can have on the quality should be investigated. Since processes like cutting can alter the chemistry of fresh plant material, reproducible methods should be developed to ensure consistent quality. Reproducible drying methods should also be developed. For example, if plant material is grown for essential oil content, any drying processes should be done carefully to preserve the volatile components. Plant material should be packaged and stored to avoid interference from pests. Conditions of storage should be carefully

chosen to preserve desired quality of the plant material, and should be well documented. Studies may be conducted to determine optimum conditions.

Cultivation of *Narcissus* as raw material for production of Galanthamine

Narcissus plants are cultivated in the Netherlands as ornamental plants. The discovery that they produce alkaloids with useful medicinal properties aroused interest in the cultivation of *Narcissus* as a medicinal crop. Cultivation of ornamental crops is different from that of crops to be consumed as food or medicine. For ornamentals yield and visual aspects of the plants are most important, and often a high input of agrochemicals is needed to satisfy these needs. Yield is also important for medicinal crops, but more important are quality aspects such as efficacy and safety. Efficacy is related to the levels of active compounds in the plant material. Safety involves the absence of toxic and potentially harmful contaminants or metabolites produced by the plant. The principles described above aim to satisfy these needs in a medicinal crop. In the following sections some aspects that may be important in the cultivation of *Narcissus* as a medicinal crop will be discussed.

Introduction to Galanthamine

Galanthamine (4a,5,9,10,1,12-hexahydro-3-methoxy-11-methyl-6H-benzofuro[3a,3,2,-ef] [2]-benzazepin-6-ol) is a natural product, belonging to the class of compounds called alkaloids. It is a benzazepine alkaloid belonging to the Amaryllidaceae group, which is found in the plant family of the same name. The structure of Galanthamine is shown in Figure 1. Galanthamine has become an important compound due to its chemical and biological properties. It is a small molecule that upon ingestion is able to cross the blood-brain-barrier and act in the central nervous system (Mucke, 1997). It inhibits the enzyme acetylcholinesterase in a long-lasting, selective, reversible and competitive manner (Moghul and Wilkinson, 2001). Acetylcholinesterase (E.C. 3.1.1.7; AChE) is one of two cholinesterase enzymes that occur in the human central nervous system. It hydrolyses acetylcholine at cholinergic synapses to terminate nerve impulse transmission. Inhibition of AChE activity increases the availability of acetylcholine to sustain nerve cell communications, and drug discovery efforts have searched for chemical inhibitors as possible leads for nervous system disorders (Lenta et al., 2008). Inhibition of AChE has been a strategy for treatment of senile dementia, ataxia, myasthenia gravis and Parkinson's disease (Mukherjee et al., 2007), but probably the most attention has been given to finding AChE inhibitors to treat Alzheimer’s disease (AD).

AD is a chronic and progressive neurodegenerative disease, and is the most common cause of cognitive decline in elderly humans (Akhondzadeh and Abbasi, 2006). Apart

from cognitive decline and memory loss, other symptoms of the disease include depression, behavioral problems and language deficits (Howes et al., 2003). Pathological features that characterize the disease are extracellular amyloid-ß-peptide deposition in senile plaques, neurofibrillary tangles, and degeneration of the basal forebrain cholinergic neurons. The loss of cholinergic neurons causes a significant reduction in acetylcholine, and this cholinergic deficit has been found to play an important role in the cognitive impairment associated with AD (Bartus et al., 1982). Increasing the levels of acetylcholine in the brain by inhibiting AChE has become the most extensively used strategy for the symptomatic treatment of AD.

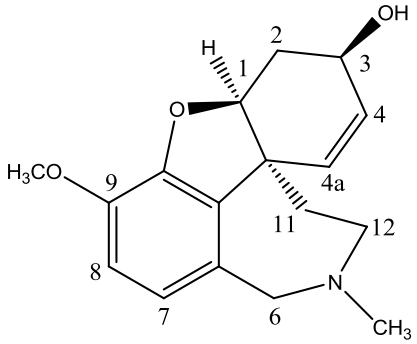

Figure 1. Chemical structure of galanthamine.

There is currently no preventative or curative treatment available for AD. Providing relief of the symptoms of the disease is the only available therapeutic option. Galanthamine is used therapeutically as the hydrobromine salt, and offers superior pharmacological profiles and fewer side effects than other AChE inhibitors such as tacrine and physostigmine (Giacobini, 1998). It causes short-term improvements in cognition, function and in general every day activities for patients with mild to moderate symptoms, but it is not known whether it is effective in the longer term, after about 6 months (Patterson et al., 2004). Apart from the AChE inhibitory activity, galanthamine also possesses other biological activities. It is known to stimulate pre-and postsynaptic nicotinic receptors which can increase the release of neurotransmitters, and possibly protect against B-amyloid toxicity (Scott and Goa, 2000; Sramek et al., 2000; Arias et al., 2004). Other pharmacological actions not directly related to AD were summarized by Bastida (2006), and include affecting membrane ionic processes, causing bradycardia or atrioventricular conduction disturbances, ability to reverse aneshesia and inhibition of traumatic shock. Galanthamine has been patented as a smoking-cessation agent (Moormann, 1997), it can relieve jet lag, fatigue syndrome, male impotence, alcohol dependance, and can reduce intraocular pressure. Furthermore

it acts as a hypotensive, has weak antimalarial activity, acts as a mild analeptic and has analgesic properties.

Galanthamine was first isolated from the snowdrop plant, *Galanthus woronowii* (Heinrich and Teoh, 2004) . The use of this plant in traditional medicine systems of the eastern European regions where the plant is native has been mentioned, but clear records of this are not easily found. It is said that a Bulgarian pharmacologist noticed people rubbing the plant on their foreheads to relieve nerve pain, and thereby decided to investigate the plant further for medicinal use (Bastida et al., 2006). The first paper on pharmacology of Galanthamine was published in 1951(Mashkovsky and Kruglikova-Lvova, 1951), which showed the AChE inhibitory activity as well as the alkaloid's ability to reverse curare-induced paralysis. In the 1950s various pre-clinical investigations into pharmacological effects of galanthamine continued, and eventually the compound was registered under the trade name Nivalin® and became commercially available in Bulgaria. At the time the main use for Galanthamine as a medicine was to treat post-poliomyelitis paralysis, and other neuromuscular diseases (Bores et al., 1996; Radicheva et al., 1996). In the 1980s researchers into AD developed the "cholinergic hypothesis", which proposed a central role of cholinergic function in the pathology of the disease (Heinrich and Teoh, 2004). In 1986 galanthamine was applied to AD for the first time (Heinrich and Lee Teoh, 2004). Promising results lead to clinical trials of galanthamine for the treatment of AD, and in 1996 an Austrian company, Synochemia Pharmazeutika launched it as Nivalin®. Later two companies, Shire Pharmaceuticals in Britain and Janssen Research Foundation in Belgium, took the development of galanthamine further and launched Galanthamine as Reminyl® in a larger number of countries (Sramek et al., 2000; Heinrich and Teoh, 2004). Since then clinical studies have continued to be done and much research attention is still focused on this alkaloid for the use in AD and other indications.

Sources of Galanthamine:

As mentioned before, galanthamine was first isolated from the plant *Galanthus woronowii*. Other members of the Amaryllidaceae family have also been found to contain this alkaloid, most notably *Leucojum aestivum* and *Narcissus* species (Heinrich and Teoh, 2004). For about 40 years, *Leucojum aestivum* was the main source of galanthamine in Eastern Europe. With the more widespread licensing of Galanthamine in the western world in recent years, alternative sources to the quite limited supply from *Leucojum* have been investigated. In the 1950s the genus *Narcissus* was investigated as a source of galanthamine, and it was found in various species (Cherkasov, 1977). Levels of the alkaloid vary between cultivars and species, from only trace amount to as much as 2.5 % of dry weight (Cherkasov and Tolkachev, 2002). A full chemical synthesis of

galanthamine was published in 1962 (Barton and Kirby, 1962), but this biomimetic method was for proof of structure and had a yield too low to be useful for commercial purposes. Since then many synthetic methods have been published to improve on the original ones (Lewis, 1998, 1999, 2000, 2001; Jin, 2003, 2005). Many methods now exist for the synthesis of galanthamine, either total synthesis or from precursors. The first patent on the synthesis of galanthamine was obtained by Sanochemia Pharmazeutika in 1996, with global protection of the process. Cooperation agreements with Janssen (Johnson & Johnson) and Shire Pharmaceuticals followed a year later (Heinrich and Teoh, 2004).

Narcissus as raw material for Galanthamine production

Recently synthetic methods have been used to produce galanthamine for the pharmaceutical industry (Tiffen, 1997). The high cost of production was acceptable because of the protection offered by licensing and patents, giving companies exclusive rights to produce the drug and so having no competition where the price is concerned. Patent protection expired recently, allowing generic products to enter the market which can bring drug prices down. Also, in 2004 about 15 million people were estimated to be suffering from AD worldwide, and this number is predicted to be three times higher in fifty years' time (Forman et al., 2004). The increasing demand and the impending price competition make other sources of galanthamine attractive. Focus has again shifted back to *Narcissus* plants, and specifically the bulbs, as a source of galanthamine. In the UK a large study into the production of Galanthamine from *Narcissus* was launched by the Department of Environment Food and Rural Affairs. The cultivation of *Narcissus* plants as raw material for the production of Galanthamine in the Netherlands is now being investigated.

Cultivation of *Narcissus* in the Netherlands

Introduction to *Narcissus*

Narcissus L. is one of 59 genera in the monocotyledonous family Amaryllidaceae (Meerow and Snijman, 1998). This genus contains about 80 to 100 wild species that originate from southern Europe and northern Africa, with a concentration of biodiversity in the region west of the Mediterranean Sea. Different *Narcissus* species are able to hybridize, and this has led to thousands of *Narcissus* cultivars being produced for commercial use. The Royal Horticultural Society in London registers and keeps track of *Narcissus* types and all cultivars are listed in the Classified List of Daffodil Names and the International Daffodil Checklist (Kington, 2008). In the Netherlands the *Koninklijke Algemeene Vereniging voor Bloembollencultuur* (K.A.V.B)

is the organization in charge of registering *Narcissus* cultivars. Different *Narcissus* types are classified according to flower characteristics into twelve groups: Trumpet, Large Crown, Small crown, Double Narcissus, Triandrus, Cyclamineus, Jonquilla, Tazetta, Poeticus, Wild species or hybrids with wild species of variants, Split crown, and all others that do not belong to any of the previous groups. The color of *Narcissus* flowers is mostly yellow, but can vary from white to pink and reddish in some cases. A color code is also given to each cultivar based on the color of the petals, eye and other flower parts.

Narcissus species are all perennial geophytes that naturally occur in a wide range of habitats. Most members of the genus flower in spring, but some do so earlier in winter and a few flower as late as autumn (Graham and Barrett, 2004). All flowers are pollinated by insects, with the most important pollinators being bees, butterflies, flies and hawkmoths (Herrera, 1995; Graham and Barrett, 2004).

Figure 2. A field of *Narcissus* plants near Lisse, The Netherlands (left), and a single plant (right).

Cultivation of *Narcissus*

Narcissus has been cultivated in the Netherlands since the sixteenth century (Hanks, 2002). Since then, cultivation has also become important in other parts of Western Europe and *Narcissus* is now one of the major ornamental spring-flowering bulb crops grown in temperate regions. It is grown commercially for the bulbs and flowers, and is also very popular in gardens, parks and other public spaces.

In terms of areas of field-grown *Narcissus* plants, the highest production occurs in the Netherlands and the UK (Hanks, 2002). Other areas where a significant amount is grown are in the Unites States, Australia and British Columbia in Canada. Israel is the main producer of the small *Narcissus tazetta* bulbs. Annual output of *Narcissus* bulbs is

estimated from the area lifted per year, the average planting density, and the average weight increase from planting to lifting. What remains after the weight required for re-planting is subtracted from the total weight of bulbs is the disposable yield. The annual disposable output for the UK and the Netherlands is estimated at about 30 000 tonnes (Hanks, 2002). A rough estimation typically gives 20 000 bulbs of medium size per ton. The bulbs are sold as planting stocks, for commercial bulb forcing (making plants produce flowers in greenhouse conditions at times when flowering does not occur naturally for cut flowers and pot plants), for amenity or landscape use, and for sale to garden centers and other outlets or for mail orders. Bulbs are also exported from the UK and the Netherlands, with the main destinations being Germany, North America and France for both countries. There is some trade between the UK and the Netherlands, with bulbs of mainstream cultivars being imported in bulk to the Netherlands, while smaller amounts of more specialized cultivars are imported to the UK. The Netherlands also imports bulbs of *Narcissus tazetta* from Israel. Not only bulbs, but also cut flowers are exported from the UK and the Netherlands on a large scale. Another important export from the Netherlands is dwarf *Narcissus* grown in pots (Heinrichs, 1999). In the growing season of 2008/2009 581 cultivars of *Narcissus* were grown in the Netherlands (PT/BKD, 1999). The top five cultivars were "Tête-à-Tête", "Carlton", "Dutch Master", "Bridal Crown" and "Ice Follies", with Tête-à-Tête accounting for 38% of the total area grown (PT/BKD, 2009).

As mentioned before, *Narcissus* has been grown in the Netherlands for several centuries. Traditionally cultivation occurred in the bulb-growing region, or *Bloembollenstreek* in the south- western parts of the country, with the town Lisse as the center. This region has sandy soil and a controlled water table, and has long been regarded as an ideal location to produce the plants (Hanks, 2002). In the last 50 years *Narcissus* cultivation has also spread to more northern locations, for example de Noord, where the soil is sandy, and also more eastwards in the country. About 1570 hectares were used for *Narcissus* cultivation in 2009 (PT/BKD, 2009), making it the third most cultivated bulb plant. The total amount of hectares grown has increased in the last few decades, but the number of growers has decreased in the same time period, thus the area grown per grower has also increased. This trend of increasing scale is mostly ascribed to increases in mechanization of farming and improvements in expertise.

Narcissus Propagation

Cultivating *Narcissus* from seed is a slow process, which takes three to six years from seed to flower. This is too long to be an efficient propagation method for large-scale commercial purposes, and is only used in breeding programs to obtain new cultivars. Vegetative propagation of *Narcissus* is also relatively slow, and on average 1.6-fold

multiplication of bulb units per year occurs. This means that 1 bulb can give rise to 1000 bulbs in about 16 years (Rees, 1969). The multiplication rate depends to a large extent on the cultivar, and factors that further affect this include growing conditions such as planting density. Lower density gives better growth and development and also more buds that grow into offsets in the same season. In the next season the new offsets will grow and come loose or be broken off. Methods such as double scaling and chipping involve dividing the bulb into pieces or segments before planting. In this way several new bulbs can develop from one original bulb.

Micropropagation, the use of single cells or groups of cells to give rise to new plants is an effective way of increasing multiplication rates. In plants usually propagated vegetatively (like *Narcissus*) micropropagation may also be the only way to obtain virus-free plant material (Sochacki and Orlikowska, 2005). Various methods and techniques have been developed for the micropropagation of *Narcissus* (Seabrook et al., 1976; Hussey, 1982; Squires and Langton, 1990). In these methods, plants were regenerated from leaf, stem, scale and stem explants, as well as tissue from the ovary wall. A drawback of using underground plant parts as explant is the higher frequency of infection with soil microbes (Seabrook, 1990).
Some methods give rise to rooted shoots that can be planted, while others produce bulblets. The bulblets have been found to survive and grow better than the shoots when planted (Seabrook, 1990). Higher sugar concentrations in the medium can increase the amount of bulblets forming in vitro (Chow et al., 1992). Not just the amount of bulblets formed, but also the size of bulblets are important, as it is related to survival upon transplanting as well as flowering (Squires et al., 1991). Different types and concentrations of sugars, as well as the osmolarity of the medium can affect the development of the bulblets in different ways (Staikidou et al., 2005). Usually bulbs formed in in vitro propagation only produce flowers after several years (Hanks, 1993). Santos' group (Santos et al., 1998) managed to produce *Narcissus* bulblets that flowered within the first year, after about 9 months of growth. Somatic embryogenesis has also been performed with *Narcissus* of various species and cultivars, using explants from various tissue types (Selles et al., 1999; Sage et al., 2000). Malik (2008) investigated micropropagation of *Narcissus* by somatic embryogenesis from ovary explants, and looked at differences between using liquid, solid or solid-liquid media. The highest biomass increase and highest number of embryos obtained directly from the explant was with an 8-week culturing system on liquid media containing growth regulators 2,4-D and BA. The results of this study also suggest good potential for liquid media in *Narcissus* for micropropagation. At present propogation of *Narcissus* in this way is feasible only at small scale, but micropropagation of *Narcissus* continues to be the focus of many investigations.

Pests and diseases of *Narcissus*:

Nematodes

The cultivation of *Narcissus* involves, like any other commercial crop, the combating of pests and diseases. The sandy soil of the *Bloembollenstreek* allows for easy spreading of nematodes. In Narcissus, *Ditylenchus dipsaci* and *Aphelenchoides subtenuis* often affect the bulbs. In the cultivated variety Carlton, other common nematode pests include *Pratylenchis penetrans* and several *Trichodorus* and *Paratrichodorus* species. The presence of nematode infestation can be seen by various symptoms. In *D. dipsaci* for example, a section through the bulb reveals dark rings as some scales turn brown upon infestation. The bulbs may also feel soft and granulose. Darkened tissue can also be seen in *A. subtenuis* infestation, especially the skins and outer scales. Another common occurrence with this nematode is the dying off of the roots, and the presence of corky tissue where the roots used to be and around the bulb base. For ornamental flower bulb purposes, infestation by nematodes can have consequences on the quality of the bulbs, and can lead to great losses or complete failure of host crops in some cases. Attack by nematodes can affect the growth of bulbs, and make them more susceptible to fungal infection and attack by the small narcissus fly. Nematodes are also vectors for certain viruses, for example Tobacco rattle virus is spread by *Trichodorus* species. Nematodes can move from diseased bulbs through the soil to healthy bulbs. The speed of movement depends on the type of soil, and is faster in sandy soils. If no host plants are in the soil the numbers of nematode rapidly decline, but usually some remain in the soil for some time. *D. dipsaci* can remain in the soil for six to sixteen years, while *A. sebtenuis* can do so for at most two years after host plants are removed.

Two strategies are used to combat nematodes through soil treatment (Rouwette et al., 2002). The first involves the use of chemical treatments. Deep-penetrating sterilizing agents such as Metam-sodium (sodium salt of methyldithiocarbamate) is a widely used agent in bulb crops, and can be used against nematodes such as *D. dipsaci* and *Trichodorus spp*. that can occur relatively deep in the soil. It may be used once every five years or more often when *D. dipsaci* has been detected (*D. dipsaci* is a quarantine organism in bulbs). Basamid strooimiddel (scattered on the ground and worked into the soil) is used together with Metam-sodium against *D. dipsaci*. This soil treatment does not work below 40 cm below the surface.

The second strategy is the practice of inundation. This involves covering the field in a layer of water for up to 10 weeks to kill harmful organisms by oxygen deprivation. This is usually done in July or August when soil temperatures are around 17 ºC. This method is effective to significantly reduce the numbers of species such as *Pratylenchus*, *Ditylechus* and *A. subtenuis*, but it works less well for *Trichodorus* nematodes. It also works against certain fungi and weeds. Since no chemicals are used, this method is

gaining popularity, and is likely to become more important as more chemicals are being banned and as resistance to allowed ones develop.
Another important method for combating nematodes is treatment of the bulbs before planting and/or after lifting with warm water. The so-called hot water treatment (HWT) against nematodes involves heating bulbs in a large water bath for four hours at 47 ºC. In the past formaldehyde was added to the water to prevent spreading of *Fusarium* and kill free-swimming nematodes. The use of formaldehyde has recently been banned due to health concerns.

Fungi
The humid conditions in the Netherlands are favorable for fungal infections of crops. Many fungal infections occur in *Narcissus*, with different varieties and species not equally susceptible to different diseases. In general fungal infections become problematic in moist conditions and warm soil and air temperatures. Cooler temperatures tend to slow or prevent fungal growth. During storage, transport and all other handling of bulbs infections can also spread easily. Fungal infections can have serious consequences on plant quality and bulb yield, and can lead to large losses in the bulb industry. These types of diseases are often latent for a time without displaying symptoms, which can make combating them more difficult.

The most common fungal disease affecting *Narcissus* is basal rot ("bolrot"), caused by *Fusarium oxysporum*. Infection starts at the bottom of the bulb and spreads upwards to the scales. Infection can also take place in wounds at all part of the bulbs. Important place is the part at the base between mother- and daughter bulb. The infected tissue becomes discolored and soft and upon drying the bulb becomes brittle. A characteristic pinkish growth of the fungus is often seen. Diseased bulbs give rise to short, twisted plants that die early. Neck rot (koprot) is also caused by *F.oxysporium*, alone or together with several other fungi. This disease has similar symptoms to basal rot except that it starts at the bulb neck and spreads down into the bulb. Infected bulbs also give rise to weak plants or no plants emerge (Hanks and Carder, 2003; Vreeburg, 2005). To prevent *Fusarium* a number of measures have to be taken. These include: A crop rotation of at most once every four years so that infection of soil is minimized, planting late so that soil temperature is less than 12°C, planting deep enough, avoiding too much nitrogen fertilization on the field, removal of diseased plants, lifting bulbs directly after harvesting foliage, drying rapidly after lifting, not washing with water after lifting, dry storage, adding fungicide while pre-soaking and to HWT, drying well after breaking bulbs, avoiding damage as much as possible and selecting and sterlizing/treating bulbs as shortly before planting as possible. For basal rot and neck rot, as for most fungal diseases, disinfection during HWT or disinfection before planting is an essential part of

control measures. The standard HWT conditions are used (2 hours at 45 ºC) and antifungal agents are added to the water bath. Alternatively bulbs can be dipped in the antifungal agents for 15 minutes in cold water.

Mechanical damage of bulbs should be avoided as much as possible, as it makes them more prone to fungal infections. Soft rot is caused by *Rhizopus arrhizus* and infection of damaged bulbs occurs at high temperatures usually during drying, at the beginning of the storage and transport of bulbs. Usually gray-brown areas develop around wound sites which turn into a soft mushy mass. Eventually a gray cobweb-like mold grows over the infected bulbs. Lowering the temperature can stop progression of the disease. Penicillium rot, as the name suggests is caused by *Penicillium* species. Disease-damaged tissue becomes brown and rotten, and this spreads to other parts of the same scale. A blue-gray to green mold is visible in infected bulbs. If the disease reaches the bulb base it may spread to other scales and spread to the whole bulb. Drying well after each damaging and disinfection HWT is done as a preventive measure, and keeping bulbs dry and warm after lifting can also prevent the development of this disease. A short period of 30°C shortly after lifting is best to prevent Penicillium rot.

Another soil fungus that may be problematic for *Narcissus* bulbs is *Sclerotinia bulborum*, which causes Black slime disease. Symptoms of the disease become visible after flowering, when the underground leaf parts become gray-brown and soft. The aboveground plant parts die down and become blackish. Bulbs can also be affected in which case the outer scale and neck turns brown and in severe cases the whole bulb disintegrates. Treatment of infected soil is necessary to prevent this disease, and is done by inundation and deep-ploughing. For bulbs a preventative measure is HWT with the fungicides, and if Black slime occurred in the field the unaffected bulbs can be given this same HWT after lifting.

For certain fungal diseases fungicide sprays are necessary to prevent severe losses or damage. Common foliar diseases of *Narcissus* include Smoulder, caused by *Botrytis narcissicola* and *Botrytis cinerea*, Fire caused by *Botrytis polyblastis* and Leaf scorch caused by *Stagonosporopsis curtisii*. These fungi produce spores that spread by wind and raindrops to aboveground foliage. Spores of *Botrytis narcissicola* and *Botrytis cinerea* can land on bulbs after lifting, and this can spread the disease to healthy bulbs. *B. polyblastis* form sclerotia on dead stems and leaves, and if in contact with the soil there germinate to produce sexual spores. Wind and rain brings these in contact with the flowers, where many asexual spores are made. This infection spreads to the stems and foliage and when the foliage dies down due to the infection, sclerotia are formed again to complete the cycle. Similarly, a heavy *Stagonosporopsis curtisii* infection can cause

the stems and leaves to die, and the fungus can survive in the dead tissue that remains behind in the field. The fungus can also come in contact with the bulbs after lifting, where it can survive in and on the bulb neck and in the bulb skins. When leaves emerge from these bulbs, they will become infected with the fungus. Specific agents are sprayed in the field for each of these diseases.
Botrytis narcissicola is responsible for another disease affecting the bulbs of Narcissus, namely type I "Vethuidigheid". This disease gives one or more of the outer scales a fatty and glossy appearance, and they later become tough and leathery with a dark brown colour. As with "Huidziek", bulb scales and skins become loose-fitting. This disease can also be caused by *Phoma leveilli*, in which case it is classified as type II. For both cases, a standard HWT with chemical additives as for *Fusarium* is the typical preventative measure. Root rot is caused by *Pythium* species and affects the roots of *Narcissus*. They develop rotten tips and bulb development is impaired, and sometimes the bulbs look like they are affected by Huidziek. Bulb sterilization with a standard HWT is used as a preventative measure, but this disease is more of a problem in potted *Narcissus* bulbs than with field-grown plants.

Some of the fungi that infect *Narcissus* bulbs in the Netherlands belong to genera that are known to produce mycotoxins. Mycotoxins are low-molecular weight secondary metabolites produced by various fungal genera including members of *Fusarium* and *Penicillium*, that can cause disease and death in humans and other vertebrates (Bennett and Klich, 2003). *Fusarium* mainly affects plants while growing in the field as a plant pathogen, and produces mycotoxins just before or after harvest, while *Penicillium* is more common as a contaminant in stages after harvest, for example during drying or storage. Some important mycotoxins produced by *Penicillium* include citrinin and patulin, while *Fusarium* are known to produce fuminosins, trichothecenes and zearalenone. Biosynthesis of mycotoxins is determined by a wide array of physical, biological and chemical factors. Time, temperature, humidity and physical damage by other organisms such as insects interact in complex ways to induce mycotoxin synthesis (D'Mello et al., 1998). Since *Narcissus* has not traditionally been cultivated for consumption or pharmaceutical use, the presence of mycotoxins has up to now not been of much importance. It is not known whether infection with *Penicillium* or *Fusarium* results in mycotoxin production in the bulbs. This may need to be investigated if *Narcissus* bulbs are to be used for pharmaceutical purposes.

Insects
The main insect pest of *Narcissus* is the larva of the large Narcissus fly (*Meredon equestris F*.). The female fly lays eggs at the foot of the plant at the beginning of summer. After a week or two larvae emerge, which then move to the bottom of the bulb

where they penetrate it. The larvae live in the bulbs from the end of June and can reach lengths of up to 18mm. In spring the larvae leave the bulb to form pupae just below the surface of the ground. In May an adult fly emerges again from the pupa to complete the cycle. Bulbs infested with these larvae often appear slightly darker in color and are less well developed. The bulbs may feel corky and often a hole is visible at the bottom of the bulbs. The larvae burrow in the bulb and make small passages, which become filled with grey granular material, and have a larva still in it after lifting. The grow points of larva-infested bulbs are usually lost, so that infested single-neck bulbs will not produce plants. Double-necked plants may emerge but generally very weak plants are formed. A standard HWT of 2 hours at 45 ºC is given as a preventative measure against narcissus fly. An additional measure has been investigated lately, namely dipping bulbs in 0.05% Admire (imidacloprid) for 15 minutes just before planting, and seemed promising against a new attack in early summer (Conijn and Korsuize, 2007). The standard HWT is also quite effective in preventing attack from other insect pests of *Narcissus* such as Narcissus mite. Slugs can attack *Narcissus* plants, but are not a very big problem and usually no action is taken against them. The small Narcissus fly is often seen during storage when bulbs are infected by *D. dipsaci*. Only diseased bulbs are infected and destroyed by the larvae of this small fly.

Viruses

Most *Narcissus* bulbs are infected with one or more viruses at any given time (Mowat, 1980). At least 24 viruses have been reported to infect *Narcissus*, but not all are commonly found (Brunt, 1995; Asjes, 1996). The presence of viruses affects the health of the plant, and can result in bulb yield losses of up to 30% (Asjes, 1996). Obtaining virus-free bulbs is difficult, and is usually only possible through micropropogation. Depending on the combination of viruses present, a variety of disorders can result, the names of which usually indicate the most dominant symptom (e.g. “Streperigheid”, “Blad bruin”, “Topvergeling”). A number of factors can affect the diagnosis of a viral disease, as certain conditions will cause some symptoms to be more pronounced. For example, long periods of cold temperatures during the growing season often cause more obvious symptoms than with higher temperatures. Other factors that can affect the degree to which viruses cause symptoms are the developmental phase of the plant, resistance of a particular cultivar, growing conditions such as soil parameters and plant density. Plants can also be infected with one or more viruses without showing symptoms at all.

Clearly the virus situation in *Narcissus* is complicated. Not much can be done to combat viruses once plants are infected, but some measures can be taken to prevent them from spreading. Growers are usually on the lookout for viral symptoms and remove seriously affected plants from the field. The symptoms for viral diseases mostly affect the leaves

and stems.

Physiological and other disorders of *Narcissus*:
Apart from attack by insects or infection by pathogens, other factors may also cause plants to be damaged. These can cause poorer quality bulbs and flowers, or complete loss of crops. Abiotic factors related to weather conditions may cause disorders, as well as treatments performed by growers during cultivation. These will be briefly discussed below.

Frost Damage:
Different *Narcissus* cultivars are not equally sensitive to frost damage, but for most types soil temperatures below -2 ºC are harmful if it continues for a few days. Light frost damage causes shorter plants and twisted leaves, with the leaves often being yellow-green with a rough appearance. The stems can become hollow and break easily. Starting from the border with the scales, the bulb base becomes glassy and gray, with a darker color if the damage is more severe. With light frost damage plants are likely to die early, and with more heavy damage bulbs will not produce plants at all. Long lasting frost can also cause a frozen ice layer in the soil that interferes with water drainage and can cause water-logging and lack of oxygen. Frost damage also makes bulbs more prone to fungal infections. To prevent heat loss from the ground a straw layer is often placed on the field after planting bulbs, or a cover crop such as wheat is planted.

Water logging:
Too much water in the soil causes roots to become rotten starting from the tips. The plants that emerge grow less well, have patches or yellowish horizontal bands on the stems and may not flower. In severe cases the roots rot away completely, and no plant is produced. The bulb tissue can become dark gray to black, but usually the bulb itself is not affected too much. Water-logging can be avoided by ensuring a good structure and drainage of the soil.

Sun scorch:
If bulbs are exposed to strong sunlight on the day of lifting or shortly after, the tissue may be damaged. When this happens the bulb tissue becomes grey-white in color, dries out and becomes very hard. The damage may go as deep as three scale layers, but may be hidden by brown bulb skins. Even if the bulbs do not seem to be damaged, plants grown from those with light sun scorch damage may show some effects. Sun scorch damage can be prevented by not lifting bulbs on very sunny days, or protecting lifted bulbs from strong direct sunlight.

Mechanical damage:
Mechanical damage of the bulbs can occur when they fall or are squashed. Damaged parts have brownish patches on the skins and under these dark gray patches ("stootplekjes") develop. The discoloration may go as deep as four scale layers. Bulbs are most vulnerable to this kind of damage during or just after lifting. The discoloration, though unsightly, usually does not affect the quality of the bulbs for flower production, although it does increase the risk of a *Botrytis* or *Penicillium* infection developing. For this reason mechanical damage should be avoided when handling bulbs by for example minimizing the falling distance in mechanized processes.

Chemical treatments:
Excessive use of herbicides in the field can have a negative effect on plants. If plants have already emerged from the bulbs, herbicides may cause gray-white, papery patches on the leaves. This damage seems to affect the bulbs as well as the aboveground parts as damage in one growing season can lead to bulbs forming plants with yellowish leaves the following year. Very heavily damaged bulbs will not produce plants the following year, or those that do emerge will die prematurely. It is not well known whether pesticides have a negative effect on the plants or bulbs.

Hot water treatment damage (*kookbeschadiging*):
HWT can cause some damage if the difference between the storage temperatures before the treatment is much lower than that of the water bath. When this happens, the tips of the roots and the tissue between the bulb base and scales become gray or black. Parts of the scales may also become gray in cases of severe damage. Plants emerging from damaged bulbs will show some effects, such as yellow patches near the tip, skew-growing leaves and dried out flower bud. In very severe cases the flower bud and stem dries out completely while still in the bulb. If a HWT is to be performed at 47 ºC against nematodes, a warm pre-treatment for one week at 30 ºC helps to reduce the damage. For the standard HWT (2 hr at 45 ºC) against nematodes, mites and flies a warm treatment is not required unless the bulbs were stored at temperatures below 20 ºC.

Quick overview of chain: how it is typically done for flower bulb production
Narcissus bulbs are planted in late summer or autumn, after which root growth from the bulb occurs rapidly. The shoot grows inside the bulbs but eventually this is slowed down by decreasing temperatures as winter approaches. Most *Narcissus* bulbs require a cold period in winter which is necessary for rapid, synchronous shoot growth and anthesis once it gets warmer (Hanks, 2002). Shoot growth is then determined by ambient temperatures and bulbs grow rapidly from around the time of anthesis. In summer leaves senesce and as a result bulb growth also slows or stops. Flowers are

harvested in spring, and the bulbs are lifted in summer. In the Netherlands an one-year cultivation system (one-year down) is used as opposed to 2 year (two-year down) system. This means that bulbs are lifted after each year, as opposed to only after two growing seasons. Two-year systems are employed mostly in non-sandy soil types, where slower growth and more difficult and expensive lifting and harvesting procedures make it more feasible to adopt a two-year system. Two year systems have some disadvantages mostly related to pests and diseases, so in the Netherlands sandy soils and ease of mechanization makes it more favorable to lift bulbs every year. A brief overview of the steps in the production chain for *Narcissus* bulbs is given as it is done for flower bulb production.

Planting stock:
The production chain starts with the planting stock. The choice of planting stock depends on the desired characteristics of the harvested bulb. Material can be obtained from auctions or private sales, or grown under contract arrangements, or from a farm's own planting stock. Bulbs are available in different grades, based on their circumference. More details on sorting and grading bulbs will be discussed later.

Bulb treatment before planting:
A standard part of the production chain is treatment of the bulbs before planting. This involves a "hot water treatment" (HWT) during which bulbs are heated in a water bath. The standard HWT involves heating bulbs at 45 ºC for 2 hours, and this is a good preventative measure against nematodes, mites and narcissus flies. Agents are added to the water bath, to help prevent certain fungal infections. This standard HWT is always done in August or September before planting in September or October. If bulbs are infected with nematodes, a longer and warmer HWT is given (47 ºC for 4 hours) and the bulbs are presoaked before the treatment. Forced bulbs always get a 4 hour treatment at 47 °C.

Soil before planting:
Before bulbs are planted, the soil should be prepared. This involves processes to ensure a good soil structure (good ratio of air, organic material, water) adequate drainage and nutrient levels. All the soil in the Netherlands is good for *Narcissus* growth and suitable for mechanization. Nematodes are however a bigger problem when different bulbs are grown every year on the same fields. Soil sterilization is sometimes part of the procedure to prepare soil for planting. This may involve chemical means or alternative methods such as inundation. Crop rotation is usually practiced in flower bulb production and usually *Narcissus* is grown on the same site once every four years.

Planting:
Narcissus bulbs are usually planted in autumn, around the end of September and in October. The advised depth of planting is 10-15 cm below the surface, and planting density depends on the type of bulbs planted. An average planting density is about 25 ton per hectare (for varieties like Carlton). When planting on sandy soil, usually bulbs are planted in beddings, in rows or covering the entire field. In heavier soils usually bulbs are plants in raised ridges.

In-field treatment:
Since *Narcissus* is prone to frost damage during the winter months, a common practice is to cover the planted bulbs with straw to keep soil temperatures from dropping too low. Sometimes a cover crop (rye, wheat) is also grown over the bulbs to achieve the same affect. Cover crops are often removed by spraying with an herbicide before the *Narcissus* emerges. Herbicide treatments are commonly employed against weeds, and are typically applied a few times during the season. Further chemical treatments in the field include pesticide sprays, especially fungicide and sometimes insecticides. This can be done preventively or diagnostically depending on the grower. Irrigation is also part of the field operations, and depends on the weather conditions during the growing season. Flower harvesting occurs in springtime.

Bulb lifting:
Bulbs are lifted from the ground in mid-July to beginning of August. Shortly before lifting the foliage is removed. Bulbs are lifted with a lifting-machine and then left in the field to dry out for a few days. Once dry enough the bulbs are picked up into bulk bins of 600 – 1200 liters, and then moved to sheds for further treatment.

Bulb handling for sale/replanting:
After lifting and drying, the bulbs are cleaned using a shaking sieve to remove soil particles. They are then transferred to a sorting machine for sorting and grading. Varieties with rough bulbs such as Carlton are sorted with spijlenplaten (plates with oblong holes) and often double-nosed bulbs are separated from round bulbs before sale. Often the stock to be sold consists of offsets and mother bulbs. Sometimes bulbs are sold per kilogram and are then not sorted. Round-holed plates are used to sort *Narcissus* with smaller bulbs such as Tête-à-Tête. Unusual or defective bulbs are removed from the bulk stock. Bulbs to be sold ("leverbaar" stock) are typically stored at about 20 ºC, at a relative humidity of 60-70% after lifting and drying. Bulbs to be planted always get a HWT. *Fusarium* infection takes time to become visible, so it is better to leave the selection of bulbs until as late as possible.

Chapter 4. Quantitative NMR method for analysis of galanthamine in *Narcissus* bulbs

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Abstract

Galanthamine is a benzazepine alkaloid used as a drug to relieve symptoms of Alzheimer's disease. For pharmaceutical use this natural product has been extracted from the plant *Leucojum aestivum (*Amaryllidaceae) or produced synthetically. Limited supply of the natural source and high cost of synthetic production has led to a search for alternative sources of galanthamine. The bulbs of *Narcissus pseudonarcissus (*Amaryllidaceae) have been identified as a potential source of raw material for galanthamine extraction. Since inconsistent chemical composition can be an issue with medicinal plant material, it is of interest to know whether large variations occur between *Narcissus* bulbs grown in different geographical locations. The goal of the study was to evaluate whether large differences exist in the overall metabolic profiles of *Narcissus* bulbs grown in the two most important cultivation regions. ^{1}H NMR and principal component analysis was used for an unbiased comparison of the bulb samples. Overall metabolite profiles were quite similar, but galanthamine levels could slightly discriminate samples by geographical region. ^{1}H NMR was used for quantitation of galanthamine, and was found to be comparable to quantitation by HPLC. Compared to conventional chromatographic methods, sample preparation for ^{1}H NMR analysis is simple and rapid, and only a small amount of plant material is required. Since both qualitative and quantitative information about the metabolic state of *Narcissus* bulbs can be obtained at the same time from the analytes by ^{1}H NMR, this method is useful for agricultural applications, and for quality control of raw material used in the pharmaceutical industry.

Introduction

As described in chapter 3, *Narcissus* bulbs contain the alklaoid galanthamine and represent a good natural source of this bioactive compound. Various methods exist for the determination of galanthamine levels in plant material. Early methods used enzyme immunoassays or radioimmunoassays for the quantitative determination of galanthamine in unpurified plant extracts (Tanahashi et al., 1990; Poulev et al., 1993). While these methods were very sensitive and precise, the need to raise antibodies and the use of radioactive substances made these methods laborious and expensive. Chromatographic methods include the use of high performance liquid chromatography (HPLC) with UV detectors (Zhang et al., 1999; Lopez et al., 2002; Mustafa et al., 2003) and mass spectrometric detectors (Ptak et al., 2008). Methods employing gas chromatography with nitrogen/phosphorus and flame ionization detectors (Bastos et al., 1996), as well as mass spectrometric detectors (Gotti et al., 2006; Berkov et al., 2008; Ptak et al., 2008) have also been developed. A recent publication describes the use of high performance thin layer chromatography (HPTLC) for determination of galanthamine in plant extracts (Abou-Donia et al., 2008). Gotti and co-workers (Gotti et al., 2006) used non-aqueous capillary electrophoresis to determine galanthamine levels in plant material. While these methods are validated to be precise and accurate, and mostly use a small amount of plant material (50-500 mg) for analysis, they all have some disadvantages inherent to the methods used. The chromatographic methods are not always reproducible between laboratories and over time. Extensive sample clean-up, long run times and consumption of large amounts of solvents can make these methods expensive and time-consuming. In addition, all these methods require the use of an analytical standard for quantitation.

Raw plant materials used for medicinal products are often inconsistent in chemical composition (Khan, 2006). This can have implications for the efficacy and safety of crude medicinal products, or the consistency of starting material for extraction of pharmaceutical products or precursors. Plants contain many metabolites (potentially more than 30,000 in a single plant) (Lay Jr. et al., 2006), and various factors are involved in determining the metabolic profile of a particular plant. Apart from its genetic profile, many biotic and abiotic factors can affect the metabolism of a plant, and thereby its metabolite composition. To understand how a plant responds to all these factors, methods are needed that can provide an overview of the full metabolic profile. Metabolomics is a promising approach to deal with quality control and other issues related to the agricultural production of medicinal plants. The metabolome can be defined as the observable chemical profile of metabolites in an organism (Verpoorte et al., 2008), and metabolomics is the qualitative and quantitative analysis of the metabolome. The large number of metabolites as well as the huge diversity in chemical

structures makes it difficult to detect and quantify all metabolites with a single analytical method. Several different approaches are currently used to analyze plant metabolites. The most common are mass spectrometry (MS) or nuclear magnetic resonance (NMR) spectroscopy-based methods in combination with multivariate statistical methods (Sumner et al., 2003; Lindon and Nicholson, 2008).

^{1}H NMR metabolomics has been successfully used in various fields of medicinal plant research, as reviewed in Chapter 2. One of the most important advantages of NMR as a metabolomics tool is the ease of quantitation, including the ease of quantitation of individual compounds in a crude mixture. Quantitative nuclear magnetic resonance (qNMR) has been used for the quantitation of natural products in various kinds of biological matrixes. Reviews by Pauli et al.(2005, 2012) cover more than two decades of literature on the topic, and some recent publications also use this method for plant secondary metabolites (Rivero-Cruz et al., 2006; Nazari et al., 2007; Castilho et al., 2008; Tatsis et al., 2008; Li et al., 2009). An advantage of qNMR over other analytical methods is elimination of the need to use analytical standards for quantitation. Also, it is relatively inexpensive in running cost, and a fast and non-destructive method requiring only small amount of plant material for analysis. Plant metabolites can be directly analyzed in a mixture without the need for fractionation and isolation. Analysis of a target compound in a mixture also provides further advantages as other metabolites (primary metabolites, non-active marker compounds) can be analyzed simultaneously (Rivero-Cruz et al., 2006) for applications such as plant identification and quality control.

In this study ^{1}H NMR-based metabolomics methods were used to investigate the chemical profiles of *Narcissus* bulbs. The aim was to investigate whether there are differences in the metabolic profile patterns of bulbs of the same species and cultivar grown in different locations, and, if so, to identify the metabolites responsible for the variation. Also, the ^{1}H NMR was applied to the quantitation of galanthamine, one of the most bioactive metabolites of *Narcissus* bulbs.

Experimental

Solvents and chemicals. Methanol-d_4 (99.80%) from Cambridge Isotope Laboratories (Andover, MA, USA) and the phosphate (KH_2PO_4) buffer (pH = 6.0) in deuterium oxide (CortecNet, Voisins-Le-Bretonneux, France) containing 0.01% trimethylsilylproprionic acid sodium salt-d_9 (TMSP, w/w), as an internal quantitation reference standard and calibration of chemical shift, were used for ^{1}H NMR analysis. Acetonitrile (HPLC-S grade), trifluoroacetic acid (TFA, reagent grade >98%) and Chromasolv® water for LC-

MS was obtained from Sigma Aldrich (St. Louis, MI, USA). Galanthamine hydrobromide was also obtained from Sigma Aldrich.

Plant material. Bulbs of *Narcissus pseudonarcissus* cv Carlton (Amaryllidaceae) were obtained from Holland Biodiversity B.V. (Lisse, The Netherlands). Bulbs were sourced from growers in two locations in The Netherlands (Lisse and Noordwijk), and one location in the UK (Lincolnshire). Ten bulbs from each location were individually frozen in liquid nitrogen and ground with a Waring laboratory blender (Waring Products Inc., Torrington, CT, USA). Ground materials were freeze-dried for 48 hours and kept at -20ºC until analysis.

NMR apparatus and measurements. ^{1}H NMR spectra were recorded using a Bruker AV 600 spectrometer (Bruker, Karlsruhe, Germany). Freeze-dried bulb material (50 mg) was transferred to a 2 mL-microtube and vortexed at room temperature for 30 s with 1.5 mL of a mixture of the phosphate buffer and methanol-d_4 (1:1). Each sample was ultrasonicated for 30 min, and centrifuged at 13000 rpm for 10 min. An aliquot of 1 mL of the supernatant was collected for ^{1}H NMR analysis. For each sample, 64 scans were recorded with the following parameters: 0.167 Hz/point, pulse width (PW) = 4.0 μs, and relaxation delay (RD) either short (1.5 s) or longer (5 s). FIDs were Fourier transformed with LB = 0.3 Hz. Manual phase adjustment and baseline correction were applied prior to integration of target regions for quantitative analysis.

Data Analysis. ^{1}H NMR spectra were automatically binned by AMIX software (v. 3.7, Biospin, Bruker). Spectral intensities were scaled to total intensity and the region of δ 0.32 – δ 10.0 was reduced to integrated regions of 0.04 ppm each. The regions of δ 4.7 – δ 5.0 and δ 3.30 – δ 3.34 were excluded from the analysis because of the residual water and methanol signal, respectively. Principal component analysis (PCA) was performed with SIMCA-P software (v.12.0, Umetrics, Umeå, Sweden) with Pareto scaling method.

HPLC measurements. HPLC analysis was done using a Waters 600E gradient controller pump and on-line Waters 991 PDA detector (Waters, Milford, MA, USA). The Vydac C18 low-TFA reversed phase analytical column (5 μm particle size, 250 x 4.6 mm i.d.) was equipped with the guard column recommended by the manufacturer (Vydac, Hesperia CA, USA). The previous method of Mustafa et al. (2003) was followed for HPLC analysis of galanthamine. Freeze-dried bulb material (200 mg) and 10 mL of 0.1% TFA (v/v) were added to a screw-top glass tube. After vortex mixing for 30 s, samples were ultrasonicated for 30 min and left overnight at 4 ºC. The following day samples were again placed in the ultrasonic bath for 30 min. A volume of 1.5 mL of sedimented supernatant was transferred to a 2 mL microtube, and centrifuged for 10 min at 13000 rpm. One mL of clear supernatant was collected for analysis, and 20 μl

was injected for analysis. The HPLC mobile phase was 10% (v/v) acetonitrile in water containing 0.1% TFA, at a flow rate of 1.0 mL/min. Galanthamine was identified by comparison of its retention time and UV-spectra with galanthamine hydrobromide standard. Quantitative measurements were performed at a wavelength of 210 nm.

Extraction recovery. A known amount of galanthamine was added to 50 mg of ground and freeze-dried bulb material. This was done by adding precise volume of a 1 mg/mL galanthamine-HBr solution in methanol-*d4* to the plant material. After addition, the samples were dried under a stream of nitrogen to dry the solvent. Once dry, the samples were extracted for NMR analysis as described above.

Accuracy of method. Solutions of galanthamine-HBr were prepared in methanol-d_4-phosphate buffer (1:1), as described above, to give a range of galanthamine concentrations from 0.039 mg/mL to 0.390 mg/mL (five different concentrations). ^{1}H NMR spectra of these solutions were recorded using two different relaxation times. Quantitative analysis was performed by integration of the area under the target signals. Calculated concentrations of galanthamine were compared to the actual concentration in solutions as weighed.

Results and Discussion

Alkaloid content is known to vary between different species of *Narcissus* (Bastida et al., 2006), but it is not known whether the same is true for the same species and cultivar grown in different geographical locations. In order to investigate the regional metabolic variation bulbs of *Narcissus pseudonarcissus* cv. Carlton obtained from two locations in The Netherlands and one location in UK were employed. These represent the most important regions for large-scale cultivation of *Narcissus* plants.

To investigate the metabolic profiles of the bulbs, a solvent was required for ^{1}H NMR analysis that would be efficient at extracting a wide range of primary and secondary metabolites. A 1:1 mixture of methanol-d_4 and KH_4PO_4 buffer in D_2O (pH 6) was chosen as this has been shown to be suitable for a wide range of metabolite extraction (Verpoorte et al., 2007). Since no reports exist of galanthamine in this particular solvent, ^{1}H NMR measurements of an analytical standard were performed and resonances were assigned as indicated in Table 1. Original ^{1}H NMR, J-resolved, COSY, and HMBC spectra are shown in Figure1 and 2.

Inspection of ^{1}H NMR spectra of bulb material extracted with the deuterated solvent showed that some of the galanthamine resonances were detected in a non-crowded region without interference from other signals in the mixture (Figure 3). To test the recovery of galanthamine with this extraction method, ^{1}H NMR measurements were

taken of three bulb samples (in triplicate) before and after adding a known amount of galanthamine to the plant material. Galanthamine was quantified by integrating the area under the singlet methoxy peak (δ 3.89) and using the area relative to the known internal standard signal area to calculate the concentration in the sample. The average recovery was calculated as 85.4% with a relative standard deviation (RSD) of 9.1% (n=9).

The bulb samples from Lisse and Noordwijk in The Netherlands and Lincolnshire in UK were analyzed by ^{1}H NMR and the recorded spectra were analyzed by principal component analysis (PCA). Using this unsupervised multivariate data analysis technique, major principal components (PCs) did not clearly distinguish between different geographical regions, suggesting that the separation of the regional

Table 1. ^{1}H NMR chemical shifts (δ)[a] and coupling constants (J) of galanthamine in the mixture of methanol-d_4 and KH_2PO_4 buffer in D_2O (1:1) (600 MHz).

Position	Chemical shift and coupling constant
H-1	4.72 (m)
H-2α	2.21 (ddd, J = 16.3, 5.3, 3.1)
H-2β	2.53 (dt, J = 16.3, 2.5)
H-3	4.28 (m)
H-4	6.07 (dd, J = 10.5, 5.0)
H-4α	6.17 (d, J = 10.5)
H-6	4.26 (dd, J = 15.2, 2.0)
H-6’	4.70 (d, J = 15.2)
H-7	6.89 (d, J = 8.5)
H-8	6.95 (d, J = 8.5)
H-11α	2.24 (dd, J = 15.5, 3.0)
H-11β	2.04 (dd, J = 15.5, 3.0)
H-12α	3.55 (dd, 13.5, 3.0)
H-12β	3.82 (td, J = 13.5, 1.0)
OMe	3.89 (s)
NMe	2.87 (s)

[a] Chemical shifts (ppm) were determined with reference to TMSP at δ 0.00

variation is smaller than biological variation. Slight separation could be observed between samples from different geographical locations only by minor principal components such as PC2 and PC4 (Figure 4a). PC2 separated the samples from Lisse from the other two sample groups, and PC4 separated the Lincolnshire samples from the Dutch groups.

Additionally, in order to examine the effect of NMR parameters on the PCA results ^{1}H NMR spectra were measured at two different relaxation times which is one of the most important parameters for quantitative features of ^{1}H NMR experiments (Pauli et al., 2005). It was evaluated whether this parameter would influence the grouping of samples in PCA analysis. In the results of PCA the difference between the spectra using different relaxation time was found to be less than the variation of the samples, that is, the

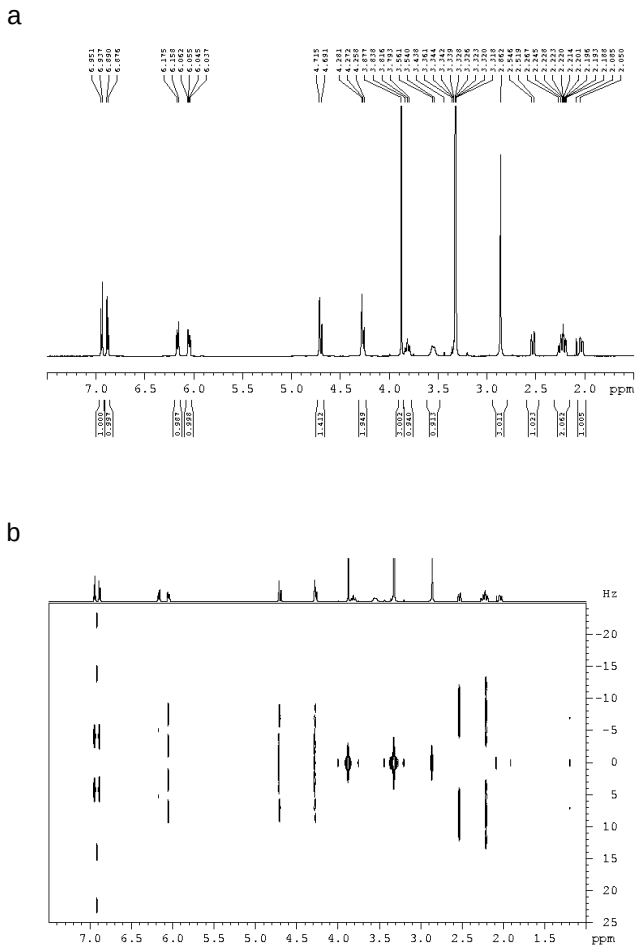
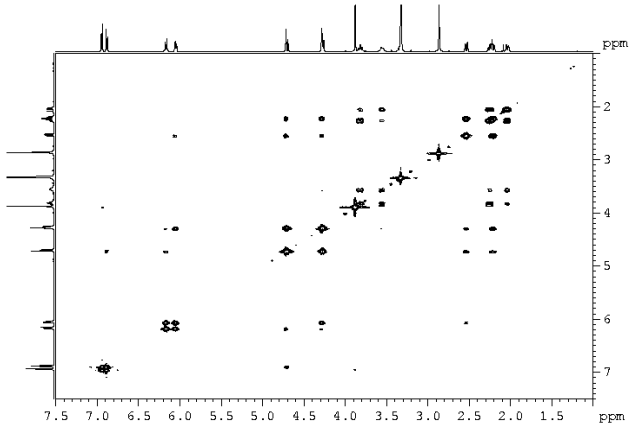


Figure 1. ^{1}H NMR spectrum (a) and J-Resolved spectrum (b) of galanthamine recorded at 600 MHz in methanol-d_4 and KH_2PO_4 buffer (pH 6.0) in D_2O, 1:1.

a



b

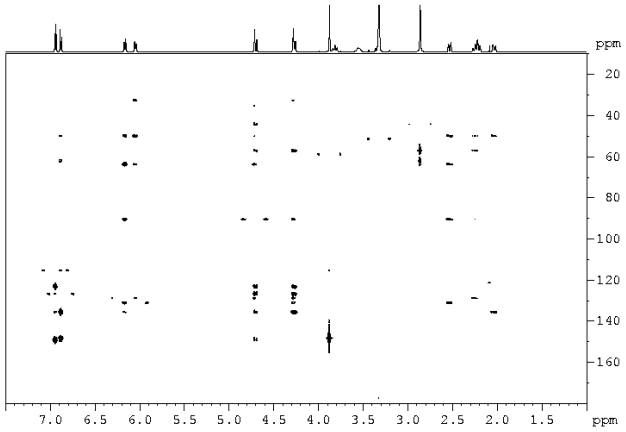


Figure 2. COSY spectrum (a) and HMBC spectrum (b) of galanthamine recorded at 600 MHz in methanol-d_4 and KH_2PO_4 buffer (pH 6.0) in D_2O, 1:1.

variation between different groups was bigger than the variation within groups of samples measured with different relaxation times. To confirm this, PCA analysis was also conducted separately with each individual geographical group and no separation of samples were seen on the basis of relaxation time in any of the groups. In Figure 4b two

kinds of ^{1}H NMR spectra obtained from different relaxation time (1.5 and 5 sec) are not separated at all.

Even though there are only minor metabolic differences between the samples employed in this study, the metabolites associated with regional variation were elucidated by loading plot. The ^{1}H NMR signals with the most influence were all found to be in the range of δ 3.2 - δ 3.9, which is the region of the spectra mostly crowded with primary metabolites such as amino acids and sugars. Interestingly, some aromatic resonances including δ 6.84, δ 7.12, δ 6.52 and δ 6.32, together with galanthamine resonances were clearly related with the regional variation of the *Narcissus* samples. The original ^{1}H NMR spectra were inspected to elucidate the resonances. The data bucket including δ 6.84 contains galanthamine signals, as shown earlier with the pure compound. The doublet signals at δ 7.13 (J = 8.4 Hz) and δ 6.74 (J = 8.4 Hz) were assigned to 4-hydroxyphenylpyruvate (4-HPP), a precursor in the galanthamine biosynthetic pathway. For the signals at δ 6.51 (J = 10.2 Hz) and δ 6.32 (J = 10.2 Hz, 5.4 Hz), the splitting pattern and 2D-correlation were similar to the signals of H-4 and H-4a of galanthamine (at δ 6.17 and δ 6.07), but occur at a different chemical shift. Haemanthamine is the second most abundant alkaloid in the *Narcissus* cultivar Carlton (Gotti et al., 2006). ^{1}H NMR spectra of haemanthamine have been reported (Bastida et al., 1987; Pabuççuoglu et al., 1989). Different NMR solvents were used in those studies so the chemical shifts and coupling constants are slightly different than in this study. However, the signal patterns are the same, and together with the fact that this is the second most abundant alkaloid in this cultivar of *Narcissus* the above-mentioned signals most likely belong to H-1 and H-2 of this alkaloid. The compounds responsible for the slight separation seen between the Lisse samples and the others were galanthamine and 4-HPP. These compound signals were negatively correlated so that samples with more galanthamine contained less 4-HPP. Since 4-HPP is one of the biosynthetic precursors of galanthamine (Bastida et al., 2006) this is not an unexpected finding. Galanthamine content was also observed to be negatively correlated to haemanthamine content.

Little variation was seen in the overall pattern of metabolites between samples from different geographical locations. However, it seemed that galanthamine levels were able to discriminate between samples together with other metabolites. The same ^{1}H NMR spectra used for the PCA analyses were used for the quantitative determination of galanthamine in the bulb extracts. One of the galanthamine resonances at δ 6.17 (doublet, J =10.15 Hz) was chosen as the target peak for quantitative analysis. Usually singlets are preferred as target peaks for quantitative NMR experiments, but since the methoxy signal of galanthamine used previously occurred in a crowded region of the spectra, it was not suitable for this purpose. Fig. 3 shows the region of the ^{1}H NMR spectra of three bulb samples containing the target galanthamine peak.

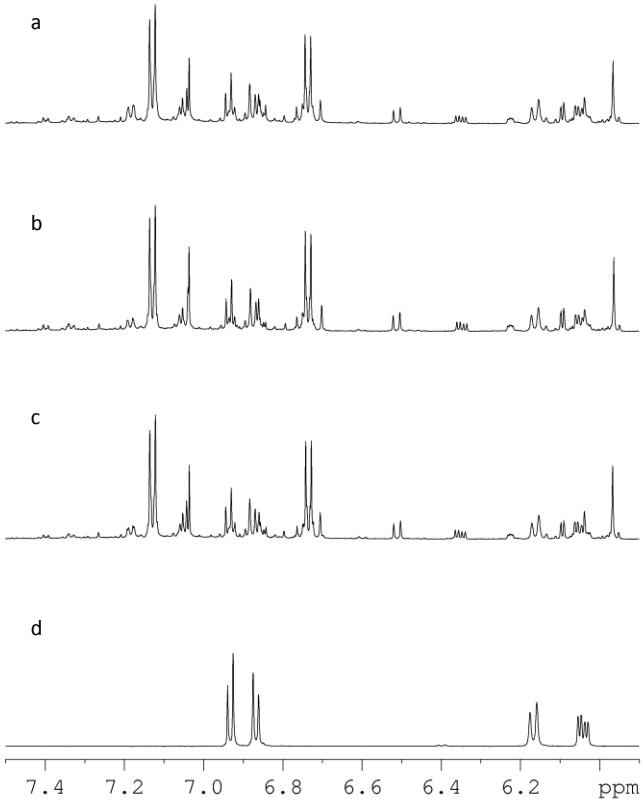


Figure 3. ^{1}H NMR spectra (600 MHz, methanol-d_4 and KH_2PO_4 buffer in D_2O, 1:1) of *Narcissus pseudonarcissus* bulb extracts obtained from Lisse in The Netherlands (A), Noordwijk in The Netherlands (B) and Lincolnshire in UK (C) together with reference compound of galanthamine HBr (D) in the range of δ 5.9 – δ 7.5.

In a previous report Pauli et al. (2005) describes the importance of optimizing the relaxation delay for qNMR experiments, as insufficient relaxation delay can result in inaccurate quantitation. To see how relaxation time affects quantitation of galanthamine, an analytical standard was dissolved in methanol-d_4 and KH_2PO_4 buffer

(1:1) at a range of concentrations for qNMR analysis. Final concentrations of galanthamine in the samples were from 0.039 to 0.390 mg/mL. Two measurements were taken at different values of d1 (relaxation delay). The ratio of the area under the target signal (6.145 to 6.185 ppm) relative to the area under the internal standard peak (TMSP, -0.11 to 0.11 ppm) was used to calculate the concentration of galanthamine. Table 2 shows the difference between the two measurements, and the longer delay time gave results closer to the actual weighed amount of galanthamine. Using the short relaxation delay, the results were found to be overestimated. It might be due to the fact that the internal standard, TMSP is not fully relaxed with short relaxation delay. The effect of relaxation delay was not detected in the PCA results because PC scores are a relative value, but the absolute quantitation results were largely affected by this parameter.

Table 2. Galanthamine content of standard solutions quantified by ^{1}H NMR, using two different relaxation delays (d1), and % difference (±) between weighed and calculated galanthamine amount.

Galanthamine (mg)	Calculated Galanthamine (mg) by d1 = 1.5s	% Difference	Calculated Galanthamine (mg) by d1 = 5s	% Difference
0.390	0.495	26.922	0.382	1.92
0.234	0.292	24.999	0.232	0.64
0.156	0.195	24.999	0.150	3.85
0.078	0.090	15.383	0.075	3.85
0.039	0.045	15.383	0.037	3.85

Galanthamine was quantified in bulb samples from Lisse and Noordwijk in The Netherlands and from Lincolnshire in UK based on 4 -5 replicates. A single ^{1}H NMR measurement was taken of each sample using the longer relaxation delay. The same bulbs samples were also extracted in triplicate and analyzed using the HPLC method of Mustafa (Mustafa et al., 2003). The results are shown in Table 3, where the galanthamine levels of the samples are quite similar to those determined by HPLC. To assess whether

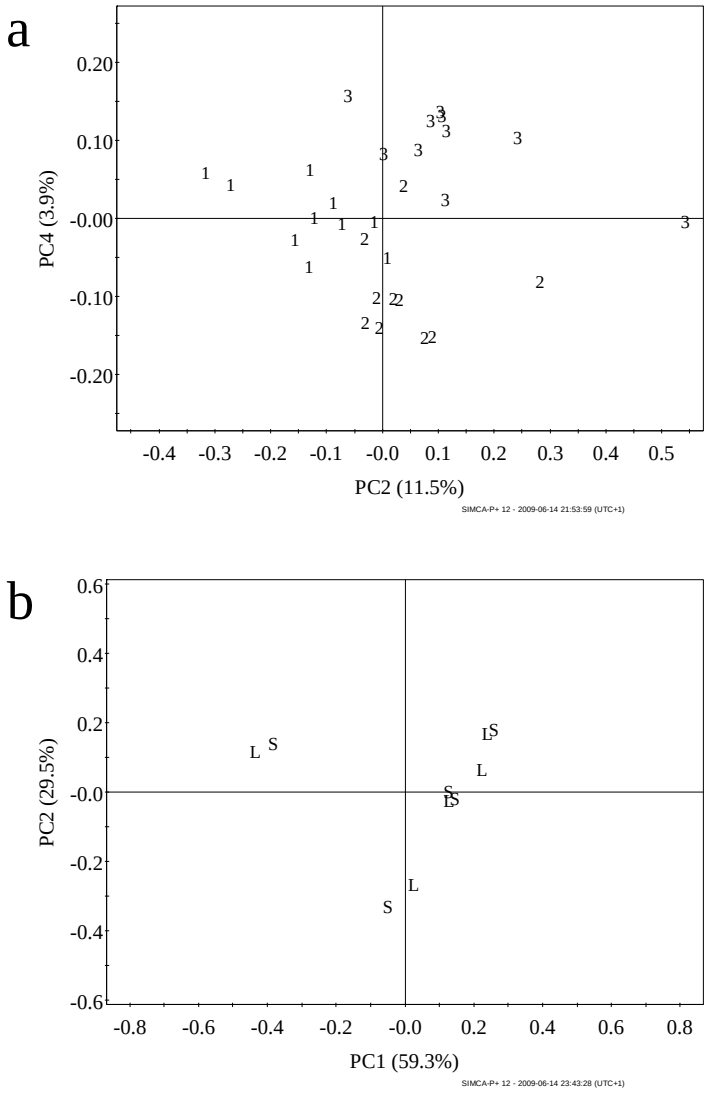


Figure 4. Score plot of principal component analysis using ^{1}H NMR spectra (PC2 vs PC4) for all the samples employed (a) and the samples obtained from Lisse, The Netherlands using two different relaxation times (b). 1; *Narcissus pseudonarcissus* bulbs obtained from Lisse in The Netherlands, 2; *Narcissus pseudonarcissus* bulb obtained from Noordwijk in The Netherlands, 3; *Narcissus. pseudonarcissus* bulb obtained from Lincolnshire in UK, S; short relaxation time (1.5 s), L; long relaxation time (5.0 s).

there was any statistically significant difference between these results a Paired Student's T-test was performed comparing the ^{1}H NMR results with the HPLC results. A P-value of 0.066 meant there was no statistically significant difference between the results ($P > 0.05$). Both in ^{1}H NMR and HPLC results, the samples obtained from Noordwijk, The Netherlands show the lowest level of galanthamine when compared to other samples. This is in accordance with the results obtained with PCA.

Table 3. Mean galanthamine content of bulb samples per dry weight determined by ^{1}H NMR and HPLC.

Sample	^{1}H NMR Galanthamine (mg/g ± SD), n=5	HPLC Galanthamine (mg/g ± SD), n=9
Noordwijk	2.36 ± 0.13	2.23 ± 0.05
Lisse	3.32 ± 0.22	3.13 ± 0.28
Lincolnshire	3.28 ± 0.26	2.98 ± 0.27

^{1}H NMR measurements together with multivariate data analysis was used to obtain a global view of metabolite profiles of *N. pseudonarcissus* cv. Carlton bulbs grown in different geographical locations. The results showed that there were no big differences between the overall metabolic profiles. Samples grown in different locations could be slightly distinguished on the basis of galanthamine content. ^{1}H NMR was used to quantify galanthamine in *Narcissus* bulb material, and was shown to be an accurate and precise method. Compared to other methods, sample preparation is simple and rapid, and only a small amount of plant material is required. A further advantage is the possibility of detecting and quantifying other metabolites besides the target alkaloid, such as primary metabolite precursors or other alkaloids. Quantifying haemanthamine in plant material usually requires analytical standards (Bastos et al., 1996; Lopez et al., 2002), and decomposition of the alkaloid may occur in some methods (Kreh et al., 1995; Gotti et al., 2006). This ^{1}H NMR method can potentially quantify the second most abundant alkaloid, haemanthamine using small amounts of plant material, and requiring no analytical standards. This method can provide useful qualitative and quantitative information about the metabolic state of *Narcissus* bulbs. The same ^{1}H NMR spectra can be used for quantitation as well as for use in multivariate data analysis such as PCA. It may be useful for agricultural applications, and for quality control of raw material used in the pharmaceutical industry.

Chapter 5. Effect of fertilizers on *Narcissus* bulbs

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Abstract

Narcissus bulbs contain the biologically active alkaloid galanthamine, and *Narcissus* is being developed as a natural source of the molecule for the pharmaceutical industry. The effect of fertilizer on galanthamine production was investigated in a field study using a ^{1}H nuclear magnetic resonance (NMR) metabolite profiling approach. Galanthamine was quantitated and major metabolites in the bulbs were identified. The application of standard fertilization levels of nitrogen and potassium caused a significant increase in galanthamine as compared to a control. Multivariate data analysis of the ^{1}H NMR data revealed that applying double the standard level of nitrogen fertilizer resulted in production of more amino acids and citric acid cycle intermediates, but not more galanthamine. The results indicated that standard levels of fertilizer currently applied in the Netherlands are sufficient for optimal galanthamine accumulation in the bulbs. This study shows how ^{1}H NMR-based metabolic profiling can provide insight into the response of plant metabolism to agricultural practices.

Introduction

Galanthamine ((4a*S*,6*R*,8a*S*)- 5,6,9,10,11,12- hexahydro- 3-methoxy- 11-methyl- 4a*H*-[1]benzofuro[3a,3,2-*ef*] [2] benzazepin- 6-ol) is an alkaloid produced by members of the Amaryllidaceae plant family. In 1952 it was first isolated from *Galanthus woronowii*, and has also been found in species in the genera *Amaryllis*, such as *Lycoris*, *Leucojum* and *Narcissus* (Cherkasov, 1977). The biological activities of galanthamine have attracted much attention and lead to its application to treat Alzheimer's disease (AD) (Sramek et al., 2000; Moghul and Wilkinson, 2001). Galanthamine has been registered as a drug (Nivalin®, Reminyl® and later Razadine®) since the mid-1990s and is used for relief of the symptoms of AD (Heinrich and Lee Teoh, 2004). Galanthamine can be produced synthetically (Czollner et al., 1998), but extraction from plants is still the major source for the pharmaceutical industry. Currently galanthamine in extracted from *Narcissus* cultivars, *Leucojum aestivum*, *Lycoris radiata* and *Ungernia victoria* in different parts of the world (Berkov et al., 2009). In the Netherlands *Narcissus pseudonarcissus*, L. is under development as a crop for galanthamine production. The cultivar Carlton was chosen as it contains galanthamine as the major alkaloid in the bulbs (Kreh et al., 1995), large stocks of bulbs are already commercially available and cultivation practices are well established due to the long tradition of ornamental use (Kreh, 2002).

The effect of nitrogen fertilization has been studied in various alkaloid-producing plants. Since alkaloids contain nitrogen and are usually derived from amino acid precursors, it has generally been assumed that higher levels of nitrogen availability would lead to higher levels of alkaloids in plants (Khan and Harborne, 1990). This was shown to be the case in studies done in the 1970s on crops such as tobacco (*Nicotiana sp.*), lupines (*Lupinus sp.*), barley (*Hordeum vulgare*), *Atropa*, *Papaver* and *Datura* (Waller and Nowacki, 1979). In more recent studies on periwinkle (*Catharanthus roseus*), yaupon (*Ilex vomitoria*) and *Datura* increased alkaloid accumulation was also observed as a result of nitrogen fertilization (Demeyer and Dejaegere, 1998; Sreevalli et al., 2004; Palumbo et al., 2007).

In *N. pseudonarcissus* cv. Carlton, fertilization increased galanthamine content in the bulbs as compared to no fertilization (Kreh, 2002). We wanted to know whether the standard level of fertilization as typically applied to increase the yield of ornamental *Narcissus* crops in The Netherlands is optimal for galanthamine production, and whether adding more fertilizers would lead to more galanthamine accumulating in the bulbs. In the study of Kreh (2002) galanthamine was determined after the bulbs were in the ground for two years. In The Netherlands bulbs are usually lifted after one year, so it

was also of interest to determine fertilizer effects on galanthamine within the typical time frame of cultivation.

In this study galanthamine in *N. pseudonarcissus* cv. Carlton bulbs treated with different levels of fertilizer was determined using a quantitative proton nuclear magnetic resonance (^{1}H NMR) method described in chapter 4 (Lubbe et al., 2010). The NMR spectra were analyzed by principal component analysis (PCA) to observe effects on metabolite profiles other than changes in galanthamine. A targeted approach using gas chromatography (GC) was also used to assess relative ratios of alkaloids in the bulbs.

Materials and methods

Chemicals and solvents

For the NMR analysis methanol-d_4 (99.80%) from Cambridge Isotope Laboratories (Andover, MA, USA), and phosphate (KH_2PO_4) buffer (pH=6.0) in deuterium oxide (CortecNet, Voisins-Le-Bretonneux, France) containing 0.01% trimethylsilylproprionic acid sodium salt-d_4 (TMSP, w/w) as an internal standard for quantitation and calibration of chemical shift was used. For GC sample preparation chloroform (analytical grade, stabilized with ethanol) from Biosolve B.V. (Valkenswaard, The Netherlands) was used. Hydrochloric acid (36.5-38.0%) and anhydrous magnesium sulphate were purchased from Sigma-Aldrich (St. Louis, MO, USA), and sodium hydroxide pellets (analytical grade) was purchased from Merck (Darmstadt, Germany). The internal standard used for the GC analysis was papaverine from BDH laboratory reagents (Poole, England).

Fertilizer treatments

Bulbs of *Narcissus pseudonarcissus* cv. Carlton were planted in November 2008 in sandy soil in Lisse, the Netherlands. For each treatment a plot of bulbs was planted, which consisted of two rows of 11, and two rows of 10 bulbs (total 42 bulbs). The rows were planted 18 cm apart, and each plot was surrounded by an edge of open space of 70 cm. Fertilizers were applied in the spring, and one plot was left untreated for a control. The standard nitrogen application was 110 kg/hectare of Kalksalpeter (Ca(NO3)2)with 19% Ca and 15.5% N, consisting of 14.4% N-NO3, 1.1% N-NH_4) applied, applied in three applications of 40 kg, 40 kg and 30 kg on 12 March, 26 March and 29 April, respectively. The double nitrogen treatment received an additional 40 kg, 40 kg and 30 kg of nitrogen fertilizer on 19 March, 2 April and 5 May, respectively. The standard potassium treatment consisted of 150 kg of Patentkali (K_2SO_4 and $MgSO_4$, 30% K as K_2O, 10% Mg as MgO and 42% S as SO_3) given in two applications of 75 kg each on 12 March and 26 March. The double potassium treatment received two extra applications of 75 kg each on 19 March and 2 April. The bulbs did not receive a hot

water treatment and were not disinfected before planting. No herbicides, fungicides or insecticides were used on the field. The bulbs were lifted at the beginning of July and were dried and stored for two weeks in the shed at 20 °C before analysis. Bulbs were inspected and 9 healthy bulbs (no fungal rot or other disease symptoms) from each treatment were selected for analysis.

Extraction of bulbs for ^{1}H NMR

Bulbs were rinsed to remove soil particles. Roots and basal plate was removed to aid cutting. Bulbs were frozen in liquid nitrogen and individually ground to fine powder in a Waring laboratory blender (Waring Products Inc., Torrington, CT, USA). Ground bulb material was freeze-dried for 7 days. Fifty mg of freeze-dried bulb material was weighed into 2 mL-microtube and extracted with a mixture of phosphate buffer (pH 6.0) and methanol-d_4 (1:1). Samples were ultrasonicated for 30 minutes, followed by centrifugation at 13000 rpm for 10 minutes. An aliquot of 800 µL transferred to NMR tubes for ^{1}H NMR measurement.

^{1}H NMR measurement

^{1}H NMR spectra were recorded with a Bruker AV 600 spectrometer (Bruker, Karlsruhe, Germany). For each sample 64 scans were recorded using the following parameters: 0.167 Hz/point, pulse width (PW) 4.0 µs and relaxation delay (RD) = 5.0 s. FIDs were Fourier transformed with LB = 0.3 Hz. Manual phase adjustment and baseline correction were applied as well as calibration with internal standard TMSP to 0.0 ppm.

Data processing

For quantitative analysis of galanthamine, integration of proton signal at δ 6.17 (galanthamine H-4a) was performed. The ratio of this integral to that of the internal standard was used to calculate the amount of galanthamine per mg material. For multivariate data analysis, ^{1}H NMR spectra were automatically binned by AMIX software (v.3.7, Biospin, Bruker). Spectral intensities were scaled to total intensity and the region of δ 0.32-10.0 was reduced to integrated regions of 0.04 ppm each. The regions δ 4.7-5.0 and δ 3.30-3.34 were excluded from the analysis because of the presence of the residual water and methanol signal, respectively. Principal component analysis (PCA) and partial Least Squared analysis (PLS) was performed with SIMCA-P software (version 12.0 Umetrics, Umeå, Sweden) with Pareto and unit variance scaling method, respectively.

Statistical analysis

All statistical analyses were performed using Sigma Plot version 11.0. A Pairwise Multiple Comparison (Tukey Test) was used for comparing the average galanthamine

levels between treatments. ANOVA of all NMR signal buckets was performed using Multiexperiment Viewer (v.4).

Gas Chromatography

Bulb material was extracted using the method described by Bastos et al. (1996). Freeze-dried bulb material (500 mg, as prepared for ^{1}H NMR measurements) was extracted in 6 mL of 0.05 M HCl solution by shaking for two hours. The extracts were centrifuged for 5 minutes at 35 000 rpm. Three mL of the supernatant was collected, and this was basified by adding 1 mL of a 0.3 M solution of NaOH. Three mL of chloroform was added and the solution mixed by vortexing for 1 minute. The solution was centrifuged for 5 minutes at 45000 rpm, and the organic (lower) chloroform layer was collected. After washing the organic layer through a Pasteur pipette containing anhydrous magnesium sulphate, 1.5 mL was transferred to a test tube and dried under vacuum in a Savant speedvac© (Thermo Scientific, Waltham, MA). The dried extracts were redissolved in 100 µL methanol containing 0.25 mg/mL papaverine as internal standard. Gas chromatography was carried out on an Agilent 6890 gas chromatographer with a FID detector and Agilent 7683 Series Injector (Agilent Technologies, Inc., Santa Clara, California). The GC was fitted with a DB-5 column (30 m x 0.25 mm x 0.25 µm). Helium was used as carrier gas with a flow rate of 1.5 mL/min. The oven temperature was programmed as follows: 200-250°C at 2.5°C/min, followed by increase of 250-270°C at 10°C/min, held at 270°C for 8 minutes. The total run time was 30 minutes. The injector and detector temperatures were 250°C and 270°C, respectively. Four µL of the samples were injected, with the injector operating in split mode (1/20). GC-MS was carried out on an Agilent 7890A GC system with a 5975C Mass Spectrometric Detector and an Agilent 7693 Autosampler (Agilent Technologies, Inc., Santa Clara, CA, USA). This GC was fitted with an HP-5 column (30 m x 0.25 mm x 0.25 µm). The flow rate, oven temperature program and injector parameters were the same as for the FID analyses. The analysis was done in scan mode (50-350 m/z).

Results and Discussion

Galanthamine content in *N. pseudonarcissus* cv. Carlton bulbs was determined using the quantitative ^{1}H NMR method described by Lubbe et al.(2010). The average galanthamine levels (mg/g DW) were calculated, and are shown in Figure 1. There was a statistically significant difference between groups as determined by one-way ANOVA ($F(3,32)=8.024$, $p=0.000$). A Tukey post-hoc test revealed that the standard nitrogen and potassium treatment (3.597±0.434 mg/g, $p=0.000$) was statistically different compared to the control treatment (2.924±0.225 mg/g). There was no statistically significant difference between the control treatment and the double nitrogen, standard potassium

treatment (3.171±0.208 mg/g, p=0.304) or the standard nitrogen, double potassium treatment (3.274±0.255 mg/g, p=0.076).

Alkaloid extracts of the bulbs treated with different levels of fertilizers (n=3) were analyzed by GC-FID to determine the relative amounts of alkaloids present. GC-MS was used for identification of the alkaloid peaks in the chromatograms (Table 1). The results of the GC analysis are shown in Figure 2. The fertilizer treatments altered the amount of alkaloids present, but the pattern of alkaloids was unchanged.

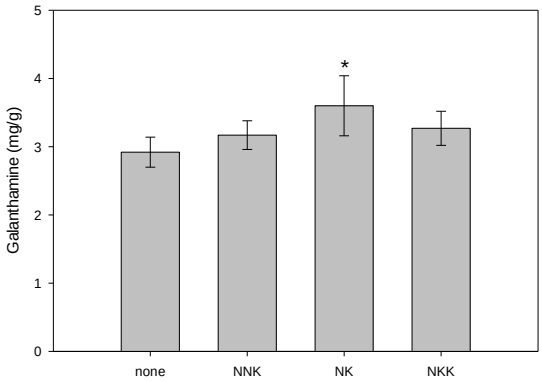

Figure 1. Average galanthamine levels of *Narcissus* bulbs treated with fertilizers compared to bulbs treated with no fertilizer (means ± SD, n=9). NNK: double nitrogen, standard potassium; NK: standard nitrogen and potassium; NKK: standard nitrogen, double potassium. *Significant at $p<0.05$.

Inspection of the ^{1}H NMR spectra showed the presence of the same metabolites in all the bulb samples, but with relative amounts differing between treatments (Figure 3). Major signals in the ^{1}H NMR spectra were assigned to primary metabolites such as sucrose, citric acid, fatty acids and various amino acids (Table 2). Signals belonging to galanthamine and haemanthamine, the two most abundant alkaloids in this cultivar of *N. pseudonarcissus* were also identified. Signal assignments in this NMR solvent system matched those previously reported (Verpoorte et al., 2007; Lopez-Gresa et al., 2010; Lubbe et al., 2010) and were confirmed using an in-house-built database of ^{1}H NMR spectra of metabolites from plants.

Table 1. GC-MS Ion Fragmentation Patterns and Retention Times of Alkaloids in Extracts of *N. pseudonarcissus* Bulbs.

Alkaloid	**RT (min)**	**$[M]^+$ and characteristic ions (% compared to base peak)**
Galanthamine	12.78	287 (83), 286 (100), 244 (24), 216 (33), 174 (28)
Lycoramine	13.00	289 (59), 288 (100), 232 (7), 202 (10), 115 (11)
Norgalanthamine	13.42	273 (99), 272 (100), 230 (35), 202 (28), 174 (13)
Epi-norgalanthamine	13.72	275 (78), 274 (100), 202 (11), 188 (18), 175 (12)
Vittatine	13.98	271 (100), 228 (22), 199 (71), 187 (66), 115 (38), 56 (18)
O-Methyllycorenine	14.71	331 (<1), 300 (3), 191 (8), 109 (100)
Lycorenine	17.21	317 (<1), 299 (6), 191 (1), 179 (1), 109 (100), 94 (3)
Haemanthamine[a]	17.82	301 (13), 272 (100), 240 (16), 211 (14), 181 (24)
Homolycorine	20.69	315 (<1), 281 (2), 207 (7), 178 (2), 109 (100), 94 (3), 82 (3)

[a]decomposition product of haemanthamine (Kreh et al., 1995).

In Figure 3 the control sample spectrum (3a) contains the same major metabolite signals as the treatments, but with the exception of sucrose they are less intense. In the standard nitrogen and potassium treatment (3b) signals assigned to galanthamine as well and other signals in the aromatic region (haemanthamine, *cis*-aconitic acid, 4-hydroxyphenylpyruvate) and fatty acid signals were increased compared to the control. These signals were also higher than the control in the double nitrogen and standard potassium treatment (3c), but appeared to be smaller than in 3b. Amino acid signals from δ 3.3-1.5 were clearly higher than in any of the other treatments. In 3d (standard nitrogen and double potassium) most metabolite signals were also higher than the control treatment, but galanthamine and other signals in the aromatic region were smaller than the standard nitrogen and potassium treatments. The amino acid signals from δ 3.3-1.5 were not as high as in 4c but appeared to be slightly higher than in the standard fertilizer treatment.

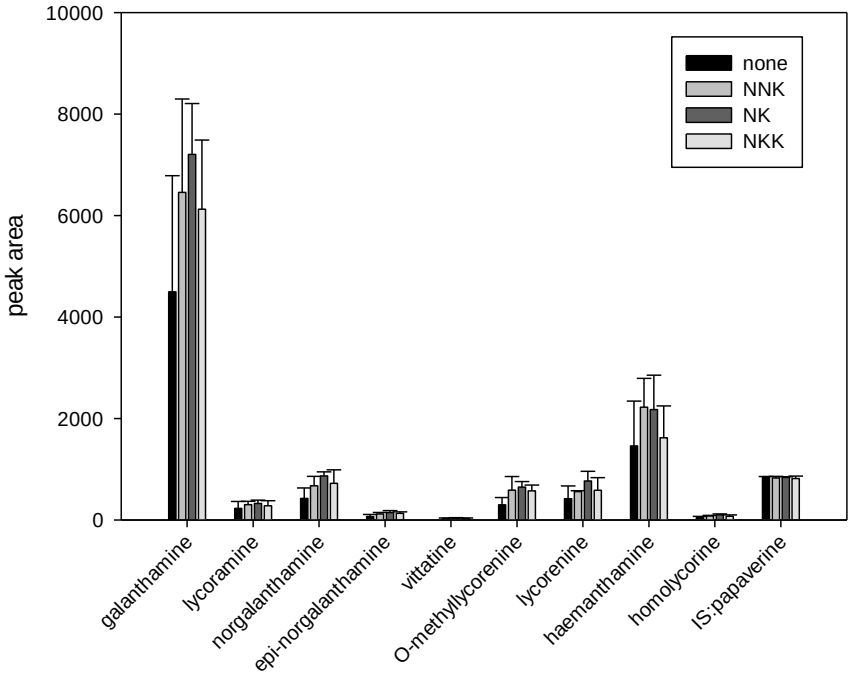

Figure 2. Average peak areas of alkaloids in GC-FID chromatograms of *Narcissus* bulbs treated with different levels of fertilizers (means ± SD, n=3). None: no fertilizer treatment, NNK: double nitrogen, standard potassium; NK: standard nitrogen and potassium; NKK: standard nitrogen, double potassium. IS: internal standard.

The differences in signal intensity between treatments were not easy to judge by visual inspection. NMR spectra can give useful qualitative and quantitative information about a sample in a single measurement, without being compound class selective (Ward and Beale, 2006). To compare the intensities of many NMR signals between different spectra simultaneously, multivariate data analysis can be used. After acquiring the spectra, they are processed to extract the data. One way is through "binning" or "bucketing", in which spectra are split into discrete regions and integrated (Schripsema, 2010). Multivariate data analysis methods such as principal component analysis (PCA) can be used to visualize the data. PCA compares all data "buckets" (variables) between samples simultaneously.

Table 2. ^{1}H Chemical Shifts (δ) and Coupling Constants (Hz) of *Narcissus pseudonarcissus* metabolites in methanol-d_4-KH_2PO_4 in D_2O (1:1) at pH 6.0.

Metabolite	Chemical shift (δ) and coupling constant (Hz)
Tyrosine	7.18 (d) J=8.4, 6.85 (d) J=8.4
4-Hydroxyphenylpyruvate	7.13 (d) J=8.4, 6.73 (d) J=8.4, 3.02 (d) J=13.6, 2.98 (d) J=13.6
cis-Aconitic acid	7.04 (s)
Galanthamine	6.94 (d) J=8.3, 6.88 (d) J=8.3, 6.16 (d) J=10.5, 6.06 (dd) J= 10.5, 5.0, 2.86 (s)
Heamanthamine	7.06 (s), 6.71 (s), 6.51 (d) J=10.3, 6.36 (dd) J=10.3, 5.0, 5.97 (brs)
Sucrose	5.41 (d) J=3.8, 4.17 (d) J=8.7, 4.03 (t) J=8.3, 3.78-3.83 (m), 3.75 (t) J=9.5, 3.66 (s), 3.51 (dd) J=9.9, 3.9, 3.43 (t) J=9.5
Glucose	4.58 (d) J=7.9, 5.19 (d) J=3.8, 3.20 (dd) J=8.8, 8.9
Choline	3.21 (s)
Asparagine	3.94 (dd) J=8.0, 4.0, 2.95 (dd) J=17.0, 3.8, 2.81 (dd) J=17.0, 8.2
Aspartic acid	2.82 (dd) 17.0, 8.5, 2.63 (dd) J=17.0, 9.5
Citric acid	2.71 (d) J=15.8, 2.56 (d) J=15.8
Glutamic acid	2.39 (td) J=7.1, 2.5, 2.10-1.28 (m)
Ornithine	3.24 (t) J=7.0, 1.92 (m), 1.65-1.78 (m), 3.71 (t) J=5.8
Alanine	1.49 (d) J=7.2
Valine	1.06 (d) J=7.0, 1.01 (d) J=7.04
Fatty acids	1.31 (brs), 0.89 (t) J=7.1, 5.40 (m)

This enables clustering of samples to reduce the dimensionality of the dataset and reduce the number of variables needed to describe it (Colquhoun 2007). Through further analysis the spectral areas responsible for grouping in the samples can be investigated, and responsible compounds can be identified.

In this study PCA was used to reduce the complexity of the ^{1}H NMR data and see what caused the maximum variance between the samples. A seven-component model explained 94.2% of the variance, with the first two components explaining 80.5%. In the score plot of PC1 and PC2 in Figure 4a, the control samples were clearly separated from the treated samples along PC1. The samples with the lowest score on PC1 (most to the left on score plot) were those treated with double nitrogen (NNK). Samples treated with the standard nitrogen (NK) had PC1 scores intermediate between control and double nitrogen (NNK). Double nitrogen (NNK) treatments were separated from the

standard nitrogen and potassium (NK) and double potassium (NKK) treatments along PC2. Samples treated with double potassium (NKK) had scores similar to the standard nitrogen and potassium (NK) samples, and were not separated from the other treatments along any of the major principal components.

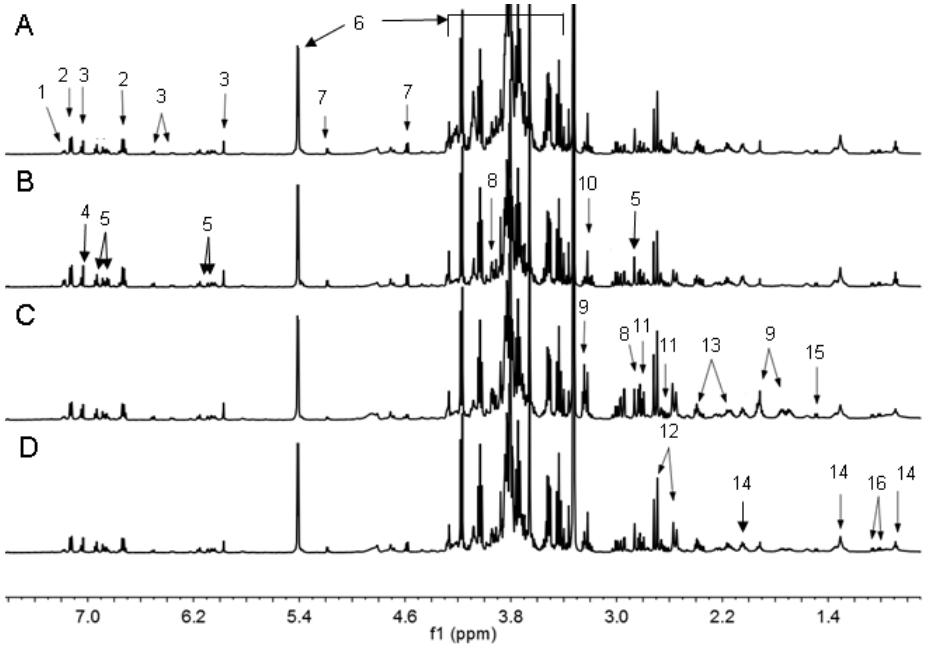

Figure 3. ^{1}H NMR spectra from δ 7.6-0.8 of bulbs treated with (**A**) no fertilizer, (**B**) standard nitrogen and potassium, (**C**) double nitrogen and standard potassium and (**D**) standard nitrogen, double potassium. 1: tyrosine, 2: 4-hydroxyphenylpyruvate, 3: haemanthamine, 4: *cis*-aconitic acid, 5: galanthamine, 6: sucrose, 7: glucose, 8: asparagine, 9: ornithine, 10: choline, 11: aspartic acid, 12: citric acid, 13: glutamic acid, 14: fatty acid, 15: alanine, 16: valine.

A loadings scatter plot of PC1 vs. PC2 (Figure 4b) shows how different areas of the spectrum contribute to the grouping of samples seen in the PCA score plot. The signals important for discriminating the control samples belong to sucrose. The signals associated with the double nitrogen treatment were mostly below δ 3.0. These signals were assigned to asparagine, aspartic acid, citric acid and ornithine. For samples that received the standard nitrogen application, ^{1}H NMR signals responsible for their position on the score plot were mostly at higher values, between δ 5.0 and δ 7.5. Signals responsible for high scores along PC2 were galanthamine, haemanthamine, tyrosine, 4-hydroxyphenylpyruvate and fatty acid signals.

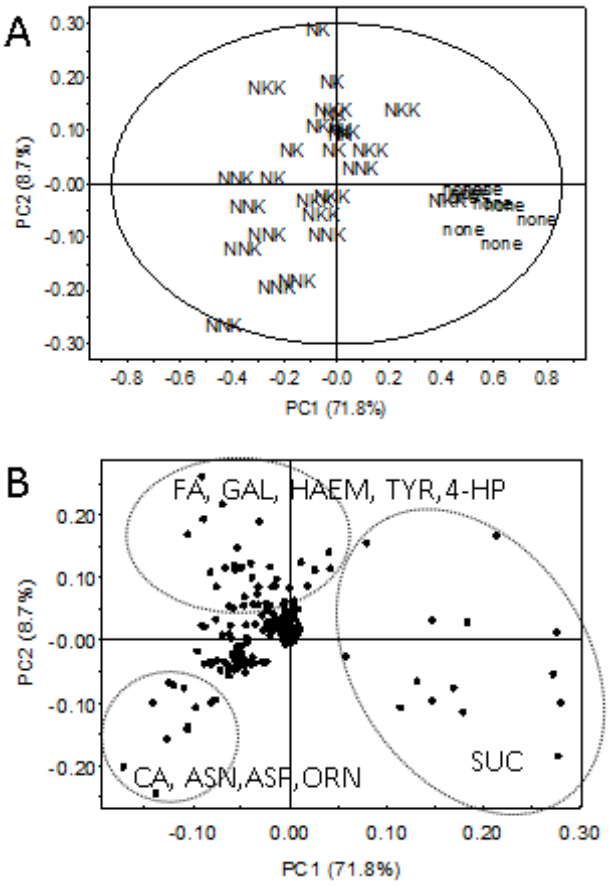

Figure 4. (A) Score plot of PCA (PC1 vs PC2) of *Narcissus* bulbs treated with different levels of fertilizers. The ellipse represents Hotelling's T2 with 95% confidence. None: no treatment; NK: standard nitrogen and potassium; NNK: double nitrogen, standard potassium; NKK: standard nitrogen, double potassium. (B) Loadings scatter plot of PCA (PC1vs. PC2) of *Narcissus* bulbs treated with different levels of fertilizers. The control (none) region of the loadings plot contains NMR signals assigned to sucrose. The standard nitrogen and potassium (NK) region of the plot contains signals of galanthamine (GAL), haemanthamine (HAEM), fatty acids (FA), tyrosine (TYR) and 4-hydroxyphenylpyruvate (4-HP). The double nitrogen and standard potassium (NNK) region of the plot contains signals of citric acid (CA), as well as the signals of aspartic acid (ASP), asparagine (ASN) and ornithine (ORN).

To determine which of the signals contributing to the PCA model were statistically different between treatments, ANOVA was performed on the integrated regions (buckets) of the NMR spectra. Figure 5 shows some of the results of the ANOVA, which confirms that the differences in metabolites seen in the PCA were statistically significant.

Optimizing the yield of galanthamine from *Narcissus* bulbs is desirable for the economically feasible extraction of this compound for pharmaceutical use. While studies have focused on increasing galanthamine production in cell or tissue culture systems (Selles et al., 1997), less is known about this in field situations. Kreh (2002) described a field test to assess the effect of fertilizers on the galanthamine content of *Narcissus* cv Carlton. It was found that applying either nitrogen or potassium/magnesium

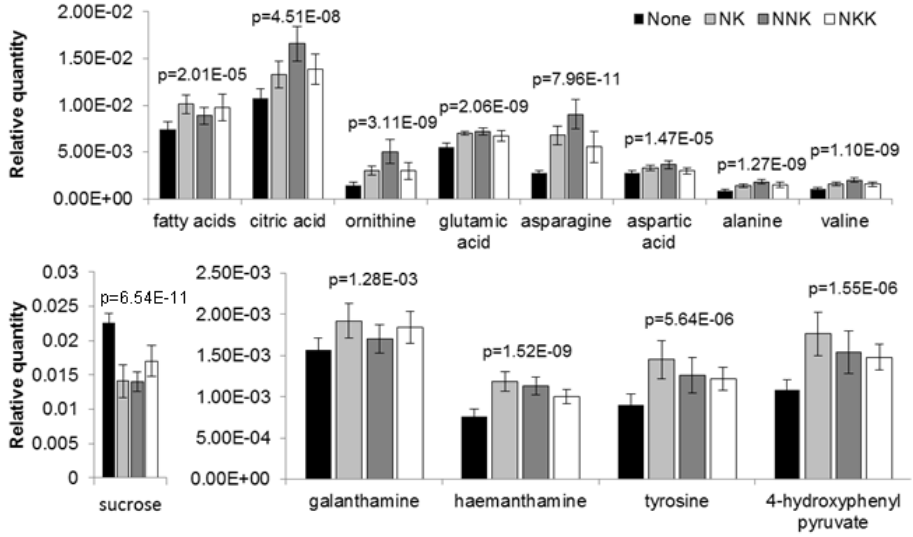

Figure 5. Relative quantification of compounds based on peak areas of associated signals (mean ± SD, n=9). Chemical shifts of signals used were fatty acids: 1.31, citric acid: 2.56, ornithine: 1.76, glutamic acid: 2.16, asparagine: 2.95, aspartic acid: 2.63, alanine: 1.49, valine: 1.06, sucrose: 4.17, galanthamine: 6.16, haemanthamine: 7.06, tyrosine: 7.18, 4-hydroxyphenylpuruvate: 7.13. The p-values of the ANOVA between the none, standard nitrogen and potassium (NK), double nitrogen and standard potassium (NNK) and standard nitrogen and double potassium (NKK) fertilizer treatment groups are shown on the graph.

fertilizer significantly increased the level of galanthamine in the bulbs compared to an untreated control. Since fertilizer was applied at one level, it is not known whether applying additional fertilizer would result in a further increase of galanthamine.
In this study, galanthamine in *N. pseudonarcissus* bulbs was lowest when the plants were not treated with fertilizer. Compared to no treatment, the standard application of nitrogen and potassium resulted in significantly higher levels of galanthamine. Double application of nitrogen and potassium both resulted in a higher level of galanthamine on average, though the difference was not statistically significant compared to the control. Alkaloid extraction and GC analysis revealed that the other minor alkaloids in the bulbs followed the same pattern, i.e. lowest levels occurred in the no fertilizer treatments, highest in the standard treatments and intermediate in the double nitrogen or potassium treatments.
The spectra of control bulbs that received no fertilizer were dominated by sucrose, which has previously been reported as the major free sugar in *Narcissus* bulb scales (Ruamrungsri et al., 1999). Nitrogen assimilation from the soil consumes carbon compounds produced from assimilated CO_2. Since less nitrogen was available to the control bulbs, it appears that more photosynthetic products were stored in the bulbs as sucrose. In the bulbs that received standard nitrogen fertilization, the availability of nitrogen throughout the season caused increased production of amino acids, fatty acids and alkaloids. Biosynthetic precursors to the alkaloids, tyrosine and 4-hydroxyphenylpyruvate, were also increased. The double nitrogen fertilizer treatment resulted in bulbs with more amino acids and citric acid than the other treatments. Increases in arginine and asparagine were previously reported in roots of *N. pseudonarcissus* upon nitrogen fertilization (Ruamrungsri et al., 2000), and was here also seen in the bulbs. The levels of alkaloids in the double N treatment were on average less than that of the standard nitrogen, although it was not significantly lower. The higher levels of amino acids indicate that the plants metabolism shifted towards producing more amino acids instead of producing more alkaloids. This is also reflected in the higher citric acid content, which is involved in the inter-conversion of amino acids and providing of precursors for many biosynthetic pathways via the citric acid cycle.
In this study adding more than the standard nitrogen fertilizer in the field during cultivation of *Narcissus* cv Carlton did not lead to production of more galanthamine in the bulbs. The application of fertilizer did increase galanthamine compared to no fertilizer treatment, but adding more than the standard amount did not lead to further increases. Applying more nitrogen-containing fertilizer does not lead to the production of more alkaloids in all plants. Luanrata and Griffin (1980) studied the effects of nutrients on a *Duboisia* hybrid (between *D. myoporoides* and *D. leichhardtii*) in hydroponic culture. Increased nitrogen application resulted in decreased hyoscyamine,

scopolamine and other alkaloid yields. In a study on the growth and alkaloid content of *Tabernaemontana*, indole alkaloid accumulation increased in response to moderate fertilization, but was not further increased upon additional fertilization (Hoeft et al., 1996).

Excessive fertilizer use is generally not recommended due to harmful effects on the environment, for example pollution of streams and lakes and increased greenhouse gas emissions (Frink et al., 1999). The use of excessive nitrogen fertilizer is also known to make bulbs more prone to infections with the fungal pathogen *Fusarium oxysporum* in *Narcissus* (Hanks and Carder, 2003; Vreeburg, 2005). The standard levels of N and K as currently applied in the Netherlands are therefore sufficient for optimal galanthamine accumulation in the bulbs. This is favorable for farmers wishing to convert *Narcissus* from an ornamental to a pharmaceutical crop, as cultivation practices do not need to be changed and fertilizer cost will not increase.

This study illustrates how ^{1}H NMR-based metabolomic profiling can be used to assess the effects of cultivation practices on plant metabolism. This method allowed the quantification of the compound of interest, galanthamine, while also providing quantitative information on metabolites of a variety of chemical classes. Primary and secondary metabolites could be identified by comparison of analyzed samples to a library of NMR spectra measured under identical conditions. In one measurement a more global view of the bulb metabolism could be obtained. PCA showed differences between fertilizer treatments, which allowed for a better understanding of the effect of the treatments on alkaloid levels.

Chapter 6. Effects of fungicides on galanthamine and metabolite profiles in *Narcissus* bulbs

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Abstract

Large-scale plant cultivation usually involves the use of pesticides. Apart from eliminating the target organism, the external chemicals may affect the metabolism of the crop plant. This may have implications for plants cultivated for specific medicinal compounds. In this study the effects of diverse fungicides on the metabolism of *Narcissus pseudonarcissus* cv. Carlton bulbs were investigated. *Narcissus pseudonarcissus* cv. Carlton is being cultivated for the extraction of the alkaloid galanthamine. Fungicides typically used in *Narcissus* cultivation were applied in a field experiment. The aim was to determine whether fungicide applications changed the concentration of galanthamine in the bulbs. ^{1}H NMR spectroscopy allowed quantitative analysis of galanthamine and other metabolites in bulb extracts. Multivariate data analysis revealed changes in bulb metabolite patterns caused by fungicides. Bulbs treated before planting generally had higher levels of alkaloids, while foliar field applications caused lower alkaloid levels but altered carbohydrate metabolism. Within these groups, certain fungicide treatments caused changes in specific metabolites. This study shows that the fungicides used in *Narcissus* cultivation can cause a change in the metabolome still detectable in the bulbs after harvest. The standard cultivation practices in terms of fungicide treatment were found suitable for the production of *N. pseudonarcissus* cv. Carlton as raw material for galanthamine extraction. In the cultivation of medicinal plants for secondary metabolites the potential effect of pesticides and other agrochemicals should be taken into account.

Introduction

Conventional large-scale plant cultivation makes use of agrochemicals that allow high crop yields, efficient use of land and minimal loss to pests. Pesticide use is sometimes associated with problems of resistance of the target organism, persistence of the active compound or its metabolites in the environment, and unwanted effects on non-target organisms (Pimentel and Edwards, 1982; Urech et al., 1997).

The crop plants to which the pesticides are applied can also be considered non-target organisms (Mitra and Raghu, 1998). There are many reports on the effects of herbicides on crop metabolism (reviewed by Lydon and Duke (1989). It is not surprising that herbicides can have an effect on crop plants, as the biochemical target of the herbicide is often common to most plants. Effects of fungicides on plant metabolism have also been reported (Werbrouck and Debergh, 1996; Ypema and Gold, 1999; Gullino et al., 2000). The aforementioned studies mainly reported the effects of fungicides on crop physiology. Several studies also investigated the effect of such agrochemicals on plant secondary metabolites. In a review on the effect of pesticides on plant secondary metabolism, Lydon and Duke (1989) summarized some effects reported in the 1970s and 1980s. These included the capacity of certain fungicides to induce phenolic compounds and other phytoalexins, enhanced lignification in infected tissues, and accumulation of antifungal compounds such as α-linoleic acid. Garcia et al. (2000) reported increased phenolic compounds in tobacco leaves with low application rates of the biocide carbendazim, and decreased accumulation at higher application rates.

Whether in the cultivation of food, medicinal or other industrial crops, it is important to know how the application of pesticides will affect the crop. In the development of new agrochemicals/pesticides, the goal is to reduce adverse effects on the crop and other organisms. Apart from pesticide residues, these agrochemicals may affect the metabolism of the plant. This effect can be assessed on different levels, such as at the level of gene expression, enzyme activity or physiological responses. The end-products of all these metabolic processes and the determining factors of the quality of the crop are metabolites. Whether it is carbohydrates and vitamins in fruit and vegetables, aromatic compounds in herbs and spices, or other secondary metabolites in medicinal plants, these compounds may ultimately be affected by anything that alters the plant metabolism.

In this study, the effects of various fungicides on the metabolism of *Narcissus pseudonarcissus* L. (Amaryllidaceae) cv. Carlton bulbs were assessed on the level of metabolites. *Narcissus pseudonarcissus* is a widely cultivated ornamental crop well-

known for the many varieties of yellow trumpet-like flowers. The bulbs of many species have been found to contain alkaloids with a number of interesting bioactivities (Bastida et al., 2006). One of these, galanthamine, has been licensed as a drug against the symptoms of early Alzheimer's disease (Heinrich and Teoh, 2004). The bulbs of *N. pseudonarcissus* cv. Carlton are currently being cultivated as raw material for the production of galanthamine. There is a long tradition of *Narcissus* cultivation in the Netherlands, which has led to optimized cultivation practices with high yields of flowers and bulbs. Ornamental flower cultivation is characterized by high input of agrochemicals. Due to climatic and soil conditions in the bulb growing region, the major diseases (e.g. bulb rot, neck rot) of *N. pseudonarcissus* are caused by pathogenic fungi (Hanks and Carder, 2003). Thus cultivation of *N. pseudonarcissus* cv. Carlton typically involves treatment of the bulbs before planting in a water bath with warm or cold water to which fungicides are added. After planting, various pesticides are applied throughout the growing season. We wanted to know if the fungicide treatments have an effect on the plant metabolism, and specifically whether they affect the galanthamine content of the bulbs. A ^{1}H-NMR-based method was used as a method to quantify galanthamine, and at the same time determine whether changes occurred in the metabolite profile of the bulbs as a whole.

Materials and Methods

Plant production and pesticide treatment

Bulbs of *Narcissus pseudonarcissus* cv. Carlton were planted on November 13, 2008 in sandy soil in Lisse, The Netherlands. A plot was planted for each treatment, and a plot consisted of two rows of 11, and two rows of 10 bulbs (total 42 bulbs). The rows were planted 18 cm apart, and each plot was surrounded by an edge of open space of about 70 cm. All plots received the standard amount of nitrogen and potassium fertilizers (150 kg K_2O Patentkali, 30% K and 110 kg KS nitrogen, 15.5% N). A control plot was planted that received no treatment at all (treatment 1: control). Treatments 2-8 were treated before planting with fungicidal agents typically used in ornamental bulb cultivation. The bulbs were dipped in a tank of cold water for 15 minutes containing one or more agrochemicals as indicated in Table 1. Treatments 10-16 consisted of fungicide treatments in the field after the bulbs were planted. These treatments were sprayed at different growth stages as is typically done in ornamental bulb cultivation. Treatment 9 consisted of bulbs treated with a mixture of fungicides before planting and in the field. A summary of all the treatments is given in Table 2. Bulbs were harvested in July 2009, and were stored and dried at 20°C for two weeks. Bulbs were kept at 4 °C for about two weeks until further processing.

Table 1. Summary of fungicide treatments applied in water bath before planting.

no.	agrochemical applied	active substance	amount active substance in water bath (% w/v)
1	No pesticide	No pesticide	None
2	Formaldehyde	Formaldehyde	1.00 (v/v)
3	Luxan	Captan	0.273
4	Sportak	Prochloraz	0.09
5	Topsin	Thiophante methyl	0.25
6	Securo	Pyraclostrobin	0.15
		Folpet	0.45
7	Shirlan	Fluazinam	0.125
8	Mix 1: formaldehyde	Formaldehyde	1.00
	Luxan	Captan	0.273
	Sportak	Prochloraz	0.09
	Topsin M	Thiophanate methyl	0.25
9	Mix 2 : formaldehyde	Formaldehyde	1.00
	Luxan	Captan	0.273
	Topsin M	Thiophanate methyl	0.25
	Securo	Pyraclostrobin	0.15
		Folpet	0.45

Sample preparation

Nine healthy bulbs were selected from each treatment for sample preparation. For the control 18 bulbs were selected. The bulbs were washed to remove soil particles and the roots and basal plate was removed to aid grinding. Bulbs were frozen in liquid nitrogen before being individually ground to a fine powder in a Waring laboratory blender. Ground bulb material was freeze-dried for 7 days. Freeze-dried material was stored at -80 °C until extraction. The plant material was extracted using the standard metabolomics method developed in our group (Kim et al., 2010). Fifty mg of freeze-dried bulb material was transferred to 2 mL microtubes and 1.5 mL of a mixture of phosphate buffer and methanol-d_4 (1:1) containing 0.01% trimethylsilylpropionic acid sodium salt-d_4 (TMSP, w/w) was added. The samples were vortexed for 30 seconds, then extracted by ultrasonication for 30 minutes. After extraction samples were centrifuged for 5 minutes, and 1 mL of supernatant was collected. Aliquots of 800 µL were transferred to NMR tubes for measurement.

Table 2. Summary of fungicides applied to *Narcissus pseudonarcissus* plants in the field.

No.	Agrochemi-cal applied	Active substance	Amount ative substance (per ha)	Time of application (number of application)
9	Full mix:			
	Maneb	Maneb	1.60 kg	Before flowering (2)
	Kenbyo	Mancozeb	0.60 kg	During flowering (2)
		Kresoxim-	0.20 kg	After flowering (3)
	Shirlan	methyl	0.40 kg	
		Fluazinam	0.19 kg	
	Rudis	Prothioconazole		
10	Shirlan	Fluazinam	0.40 kg	Before flowering (2)
				During flowering (2)
				After flowering (3)
11	Allure	Prochloraz	0.21 kg	Before flowering (2)
		chlorthalonil	0.67 kg	During flowering (2)
				After flowering (3)
12	Maneb	Maneb	1.60 kg	Before flowering (2)
				During flowering (2)
				After flowering (3)
13	Kenbyo	Kresoxim-	0.20 kg	Before flowering (2)
		methyl	0.60 kg	During flowering (2)
		Mancozeb		After flowering (3)
14	Chlorthalonil	Chlorthalonil	1.00 kg	Before flowering (2)
				During flowering (2)
				After flowering (3)
15	Rudis	Prothioconazole	0.19 kg	Before flowering (2)
				During flowering (2)
				After flowering (3)
16	Field mix:			
	Maneb	Maneb	1.60 kg	Before flowering (2)
	Kenbyo	Mancozeb	0.60 kg	During flowering (2)
		Kresoxim-	0.20 kg	After flowering (3)
	Shirlan	methyl	0.40 kg	
		Fluazinam	0.19 kg	
	Rudis	Prothioconazole		

^{1}H NMR measurement

^{1}H NMR spectra were recorded using a Bruker AV 600 spectrometer (Bruker, Karlsruhe, Germany). For each sample, 64 scans were recorded with the following parameters: 0.167 Hz/point, pulse width (PW) = 4.0 μs and an optimized relaxation delay (RD) of 5 s (Lubbe et al., 2010).

Data processing and analysis

^{1}H NMR spectra were Fourier transformed (LB = 0.3 Hz). Manual phase adjustment and baseline correction were applied as well as calibration with internal standard TMSP to δ 0.0. For quantitative analysis of galanthamine, integration of proton signal at δ 6.17 (galanthamine H-4a) was performed. The ratio of this integral to that of the internal standard was used to calculate the amount of galanthamine per mg material. For multivariate data analysis, ^{1}H NMR spectra were automatically binned by AMIX software (v.3.7, Biospin, Bruker). Spectral intensities were scaled to total intensity and the region of δ 0.32-10.0 was reduced to integrated regions of 0.04 ppm each. The regions δ 4.7-5.0 and δ 3.30-3.34 were excluded from the analysis because of the presence of the residual water and methanol signal, respectively. Principal component analysis (PCA) was performed with SIMCA-P software (version 12.0 Umetrics, Umeå, Sweden) with Pareto scaling method. Analysis of variance (ANOVA) followed by Pairwise Multiple Comparison (Tukey Test) was performed to compare average galanthamine levels between treatments using SPSS (version 18, PASW Statistics, Chicago, IL, USA).

Results

Galanthamine quantification

Galanthamine concentration in the *Narcissus* bulbs was determined using a quantitative ^{1}H NMR method developed previously (Lubbe et al., 2010). The area under the doublet signal at δ 6.16 belonging to H-4a of galanthamine was used for quantitation. Figure 1 shows the average concentration of galanthamine per treatment. A one-way ANOVA revealed that a statistically significant difference in the average galanthamine concentrations between the groups ($F(15,132) = 21.54$, $p=0.000$). A Tukey *post-hoc* test revealed that two of the treatments before planting (treatments 3 and 4) had significantly lower levels of galanthamine (2.676 ± 0.463, $p=0.036$ and 2.248 ± 0.363 mg/g, $p=0.000$, respectively) than the control treatment (3.226 ± 0.221 mg/g). Field fungicide treatments that also had significantly lower levels of galanthamine were treatment 12 (1.591 ± 0.686 mg/g), treatment 13 (2.013 ± 0.603 mg/g, $p=0.000$), treatment 14 (1.618 ± 0.739 mg/g, $p=0.000$), treatment 15 (1.315 ± 0.660 mg/g, $p=0.00$) and treatment 16

(1.837 ± 0.683 mg/g, p=0.000). Although not statistically significant, the highest average galanthamine concentration was in treatment 9 (3.542 ± 0.199 mg/g) where mixtures of fungicides were applied before planting and in the field.

Metabolite identification

The ^{1}H NMR spectra were visually inspected and signals were assigned to various primary and secondary metabolites (Table 3, Figure 2). Identification of metabolites was done with the aid of two-dimensional NMR experiments (COSY, J-Resolved and HMBC), as well as comparison of signals with an in-house metabolite database and previously reported data (Verpoorte et al., 2007; Kim et al., 2010). In all samples, spectra were dominated by signals assigned to the disaccharide sucrose (Figure 2a).

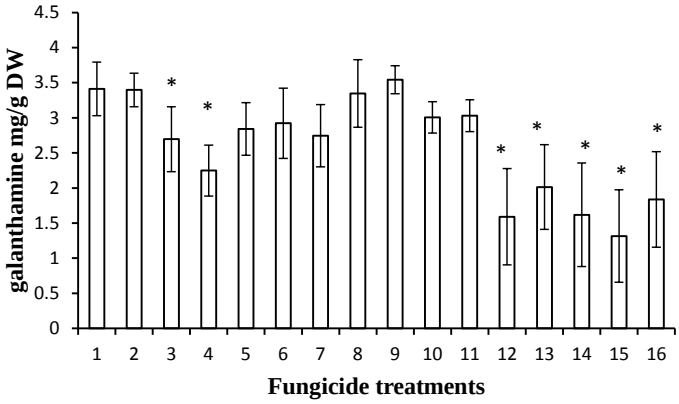

Figure 1. Average galanthamine content of *Narcissus pseudonarcissus* cv. Carlton bulbs treated with fungicides (mean ± SD, *P<0.05). Treatment 1: control (n=18), treatment 2-8: pre-planting fungicide treatments (n=9), treatment 9: pre-planting and in field fungicide treatment (n=9), treatment 10-16: in-field fungicide treatment (n=9). Treatment numbers are indicated in Table 1 and 2).

Glucose was identified by the doublet signals at δ 4.58 (J=7.9) and δ 5.19 (J=3.8) of the β- and α-anomeric protons, respectively. A doublet at δ 5.17 (J=3.8) was assigned to the H-1 of maltose with the help of COSY and HMBC experiments. Other minor sugars previously reported in *N. pseudonarcissus* bulbs (Ruamrungsri et al., 1999), raffinose and arabinose, were also identified. Signals belonging to galanthamine and other major alkaloids of *N. pseudonarcissus* cv. Carlton, as well as some biosynthetic precursors were identified in the region of δ 6-8 (Figure 2b). Galanthamine and haemanthamine signals were assigned as previously reported (Lubbe et al., 2010). The third most

abundant alkaloid narciclasine was identified with the help of two-dimensional NMR experiments and comparison of signals to the reports in the literature (Evidente, 1991). Two precursors in the alkaloid biosynthetic pathway in *Narcissus*, tyrosine and 4-hydroxyphenylpyruvate (4-HPP) were also identified (Bastida et al., 2006) (Figure 3).

Characteristic signals of various primary metabolites were seen at lower chemical shifts. This included the amino acids asparagine, aspartic acid, glutamic acid, ornithine, alanine, valine and threonine. The organic acids malic acid and citric acid were identified, as well as signals belonging to choline and ethanolamine. Characteristic triplet signals of fatty acid ω-protons were seen at δ 0.88, as well as the broad signal at δ 1.31 of their $(CH_2)_n$ chains. COSY and J-Resolved experiments also allowed identification of a triplet at δ 2.18 and multiplet at δ 1.56 of the -$CH_2\underline{CH_2}$COOH and -$\underline{CH_2}CH_2$COOH protons, respectively. In some samples a multiplet signal was seen at δ 5.40, partly overlapped with the large doublet of sucrose. This was assigned to the olifenic protons of unsaturated fatty acids, such as linoleic acid.

Table 3. ^{1}H Chemical Shifts (δ) and Coupling Constants (Hz) of *Narcissus pseudonarcissus* bulb metabolites in CH_3OH-d_4-KH_2PO_4 in D_2O at pH 6.0.

no.	Metabolite	Chemical shift (δ) and coupling constant (Hz)
1	Lycorenine	7.06 (s), 7.04 (s), 6.02(s), 5.73 (brs)
2	Phenylalanine	7.42-7.33 (m), 3.09 (dd) J=8.3, 14.8
3	Tyrosine	7.18 (d) J=8.4, 6.85 (d) J=8.4
4	4-Hydroxyphenylpyruvate	7.13 (d) J=8.4, 6.73 (d) J=8.4, 3.02 (d) J=13.6, 2.98 (d) J=13.6
5	*Cis*-aconitic acid	7.04 (s)
6	Galanthamine	6.94 (d) J=8.3, 6.88 (d) J=8.3, 6.16 (d) J=10.5, 6.06 (dd) J= 10.5, 5.0, 2.86 (s)
7	Haemanthamine	7.06 (s), 6.71 (s), 6.51 (d) J=10.3, 6.36 (dd) J=10.3, 5.0, 5.97 (brs)
8	Narciclasine	6.22 (m), 6.09 (d) J=4.5, 6.73 (s), 4.39 (m), 4.32 (m)
9	Raffinose	5.55 (d) and 5.27 (d) J=3.8 (both COSY to 3.52)
10	Sucrose	5.41 (d) J=3.8, 4.17 (d) J=8.7, 4.03 (t) J=8.3, 3.78-3.83 (m), 3.75 (t) J=9.5, 3.66 (s), 3.51 (dd) J=9.9, 3.9, 3.43 (t) J=9.5
11	Mannose	5.14 (d) J=1.5
12	Maltose	5.17 (d) J=3.8, 5.40 (d) J=3.9
13	Arabinose	5.23 (d) J=3.8
14	Rhamnose	5.11 (d) J=1.5, 4.87 (d) J=0.7, 1.28 (t) J=6.5
15	Glucose	4.58 (d) J=7.9 (β-anomer), 5.19 (d) J=3.8 (α-anomer), 3.20 (dd) J=8.8, 8.9
16	Choline	3.21 (s)
17	Ethanolamine	3.12 (t) J=5.3
18	Asparagine	3.94 (dd) J=8.0, 4.0, 2.95 (dd) J=17.0, 3.8, 2.81 (dd) J=17.0, 8.2
19	Aspartic acid	2.82 (dd) 17.0, 8.5, 2.63 (dd) J=17.0, 9.5
20	Citric acid	2.71 (d) J=15.8, 2.56 (d) J=15.8
21	Malic acid	2.68 (dd) J=15.7, 3.4, 2.36 (dd) J=15.7, 10.4, 4.28 (dd) J=10.4, 3.2
22	Glutamic acid	2.39 (td) J=7.1, 2.5, 2.10-1.28 (m)
23	Ornithine	3.24 (t) J=8.0, 1.92 (m), 1.65-1.78 (m), 3.71 (t) J=5.8
24	Acetic acid	1.91 (s)
25	Alanine	1.49 (d) J=7.2
26	Valine	1.06 (d) J=7.0, 1.01 (d) J=7.0
27	Threonine	1.34 (d) J=6.6, 4.22 (m),
28	Fatty acids	1.31 (brs), 2.18 (t) J=7.4, 1.56 (m), 0.88 (t) J=7.4, 5.40 (m)

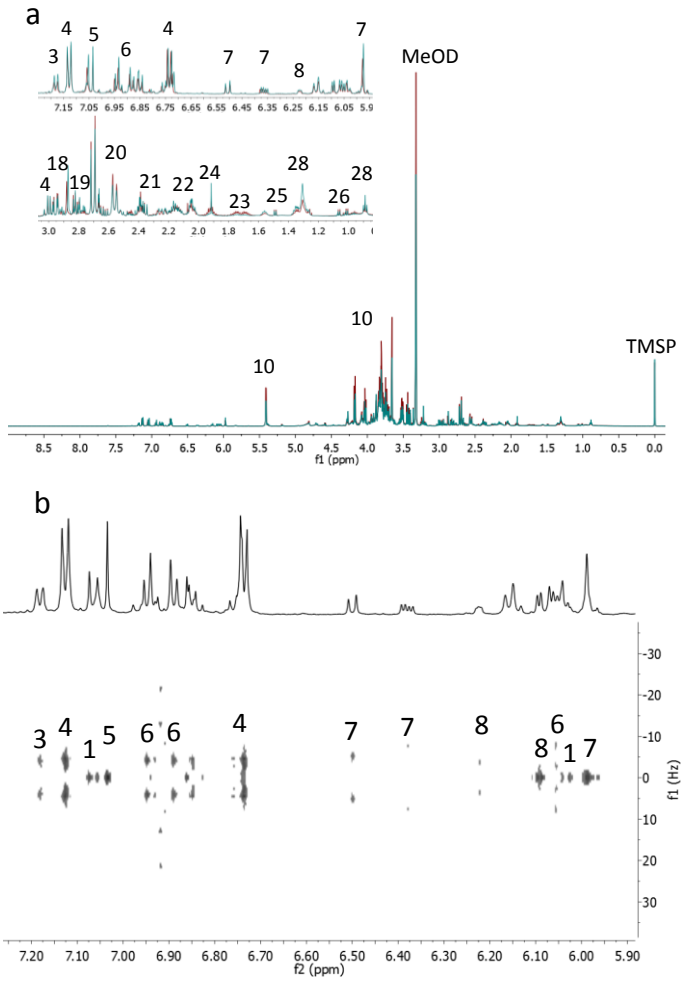

Figure 2. (a) Representative ^{1}H NMR spectra of *Narcissus pseudonarcissus* cv. Carlton bulbs of treatment 1 (control; red) and treatment 9 (fungicide mix 2; blue) extracted with phosphate buffer and methanol-d_4 (1:1), pH 6.0, showing metabolites occurring in different parts of the spectrum. 3: Tyrosine, 4: 4-Hydroxyphenylpyruvate, 5: *cis*-Aconitic acid, 6: Galanthamine, 7: Haemanthamine, 8: Narciclasine, 10: Sucrose, 18: Asparagine, 19: Aspartic acid, 20: Citric acid, 21: Malic acid, 22: Glutamic acid, 23: Ornithine, 24: Acetic acid, 25: Alanine, 26: Valine, 28: Fatty acids. (b) J-Resolved spectrum of the aromatic region of a representative *Narcissus pseudonarcissus* bulb extract. 1: Lycorenine, 3: Tyrosine, 4: 4-hydroxyphenylpyruvate, 5: *cis*-Aconitic acid, 6: Galanthamine, 7: Haemanthamine, 8: Narciclasine.

Multivariate data analysis

Multivariate data analysis was used to compare all the signals of all the ^{1}H NMR spectra. An unsupervised method, principal component analysis (PCA) was used to obtain an overview of the differences between bulb treatments in an unbiased way. PCA resulted in a model in which the first two principal components accounted for more than 70% of the variance in the dataset. The score scatter plot of the first two principal components is shown in Figure 4a. Each point in the score scatter plot represents one bulb sample. When the points in the score scatter plot are colored according to whether the treatment was applied before planting or after planting, a pattern emerges. Samples treated with fungicides before planting were roughly separated from those treated in the field along PC1, with the control samples clustered in the middle of the plot. Samples of treatment 9 grouped with the other samples that were treated with fungicides before planting.

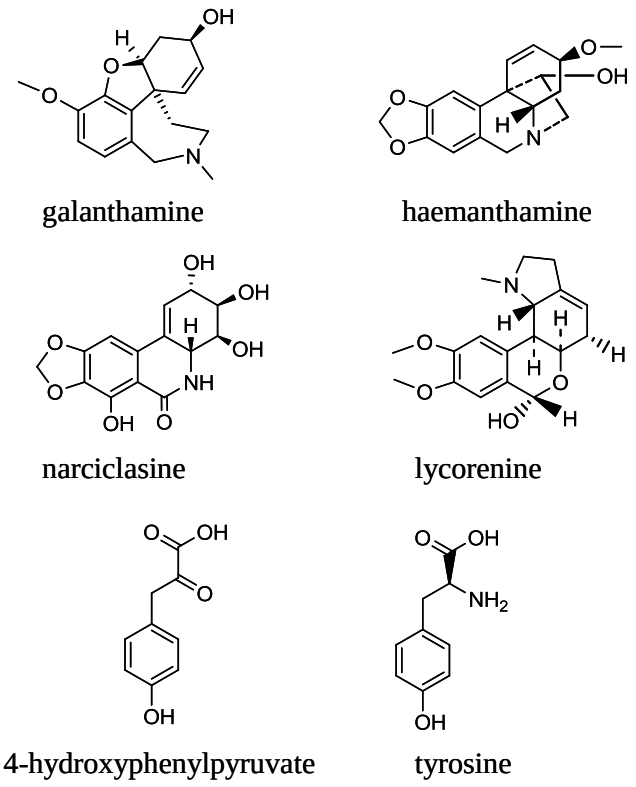

Figure 3. Chemical structures of some metabolites identified in *Narcissus pseudonarcissus* cv. Carlton bulbs.

A loading scatter plot of PC1 versus PC2 (Figure 4b) shows how different variables (buckets) contribute to the grouping of samples seen in the PCA score plot. Variables with a high positive loading on PC1 included buckets with signals assigned to sucrose, glucose and maltose (e.g. 3.80, 3.84, 3.64, 4.16, 3.44, 4.04, 3.68, 5.40, 3.36, 3.48 and 5.20). Variables with negative loadings on PC1 included buckets with signals assigned to alkaloids galanthamine (2.88) and haemanthamine (6.72), alkaloid precursors 4-HPP (3.00, 7.12) and tyrosine (6.88), amino acids aspartic acid (2.68) and glutamic acid (2.16, 2.40), organic acids citric (2.56, 2.72), malic (4.28, 2.36) and *cis*-aconitic acid (7.04) as well as fatty acids (1.36, 1.32, 0.88).

PCA was applied to those treatments which had a significantly lower concentration of galanthamine to see if any other changes in the metabolite profile might help explain the observed effect. PCA of controls and samples treated with captan (treatment 3) before planting gave a score scatter plot where the two groups were separated along PC1, with 42.7% of the variance accounted for (Figure 5a). From a loading column plot of PC1 the signals responsible for the groupings could be seen (Figure 6a). Signals with a negative loading included those of the alkaloids galanthamine, haemanthamine and narciclasine. Other signals with negative loadings on PC1 were those of tyrosine, aspartic acid, malic acid, citric acid, acetic acid and fatty acids.

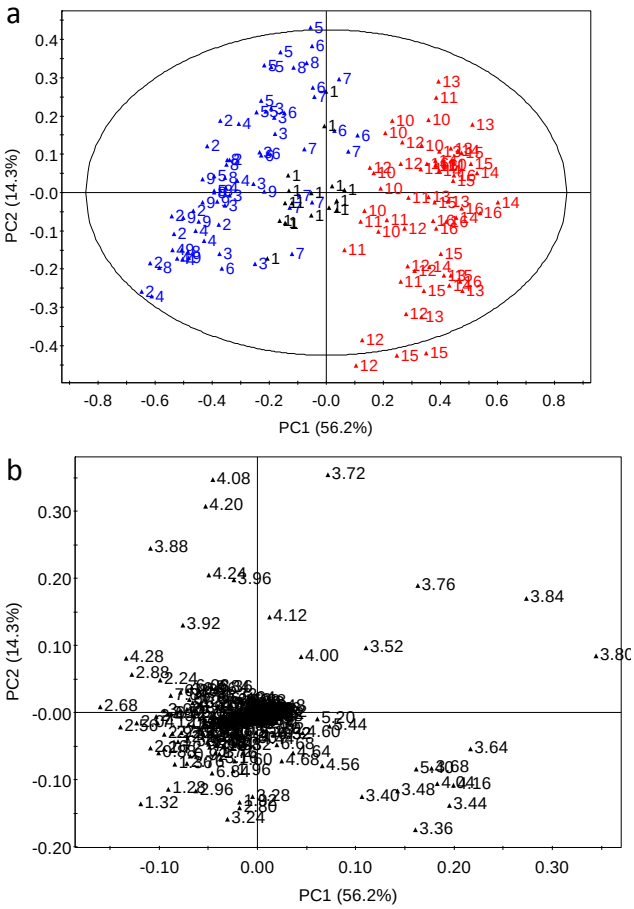

Figure 4. (a) Score scatter plot showing results of the PCA with all bulb samples. The numbers correspond to fungicide treatments as indicated in Table 1 and 2. (b) Loading scatter plot of PC1 versus PC2 of bulb samples treated with fungicides.

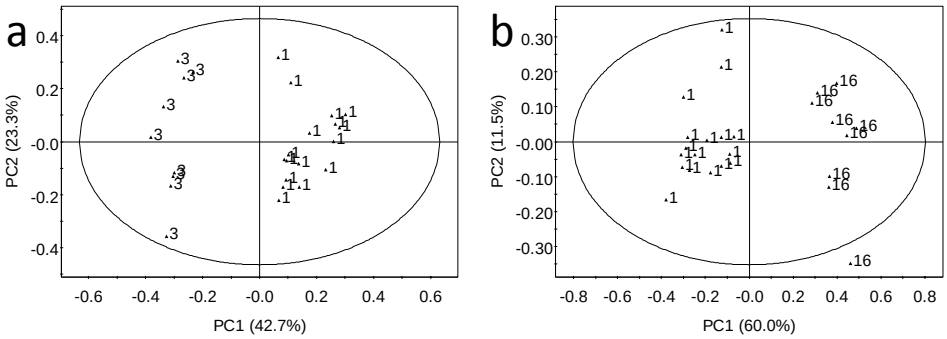

Figure 5. (a) Score scatter plot of PCA of control samples versus captan (treatment 3). (b) Score scatter plot of control samples versus field fungicide mix (treatment 16).

PCA of samples treated with prochloraz (treatment 4) before planting were separated from controls along PC1, with 38.4% of the variance explained. The treated samples were less tightly clustered and one outlier was seen outside the Hotelling's T range ellipse. Similar to the captan samples, signals responsible for the separation along PC1 were identified as the alkaloids (galanthamine, haemanthamine and narciclasine) as well as the alkaloid precursors tyrosine and 4-HPP. From the loading column plot further signals responsible for the separation in the score scatter plot were malic acid, citric acid, aspartic acid, acetic acid and ethanolamine. Two triplet signals (δ 1.19 J=7.0 and δ 1.12 J=7.4), which also contributed to the separation of the samples were observed in the prochloraz treatments but not in the controls. They were tentatively assigned to the methyl group of 1-*O*-ethyl-β-glucoside and terminal methyl group of a fatty acid, respectively. A double doublet signal at δ 5.50 (J=7.0, 3.5) also had a negative loading on PC1.

Treatments 12-16 were also submitted individually to PCA to compare their metabolite profiles with the control samples. In each case separation was seen between the treated samples and the controls along PC1 in a score scatter plot, with the first PC explaining between 40 and 60% of the variance. The variables responsible for the separation were similar in these field fungicide treatments. Figure 5b shows treatment 16 as an example. Signals common to all were those belonging to sucrose, the unsaturated fatty acid signal at 5.44, the dd at δ 5.50, fumaric acid, glucose and maltose (Figure 6b). In the maneb, rudis and field mix treatments formic acid contributed to the loading, and in chlorothalonil, rudis and field mix treatments raffinose had a positive loading.

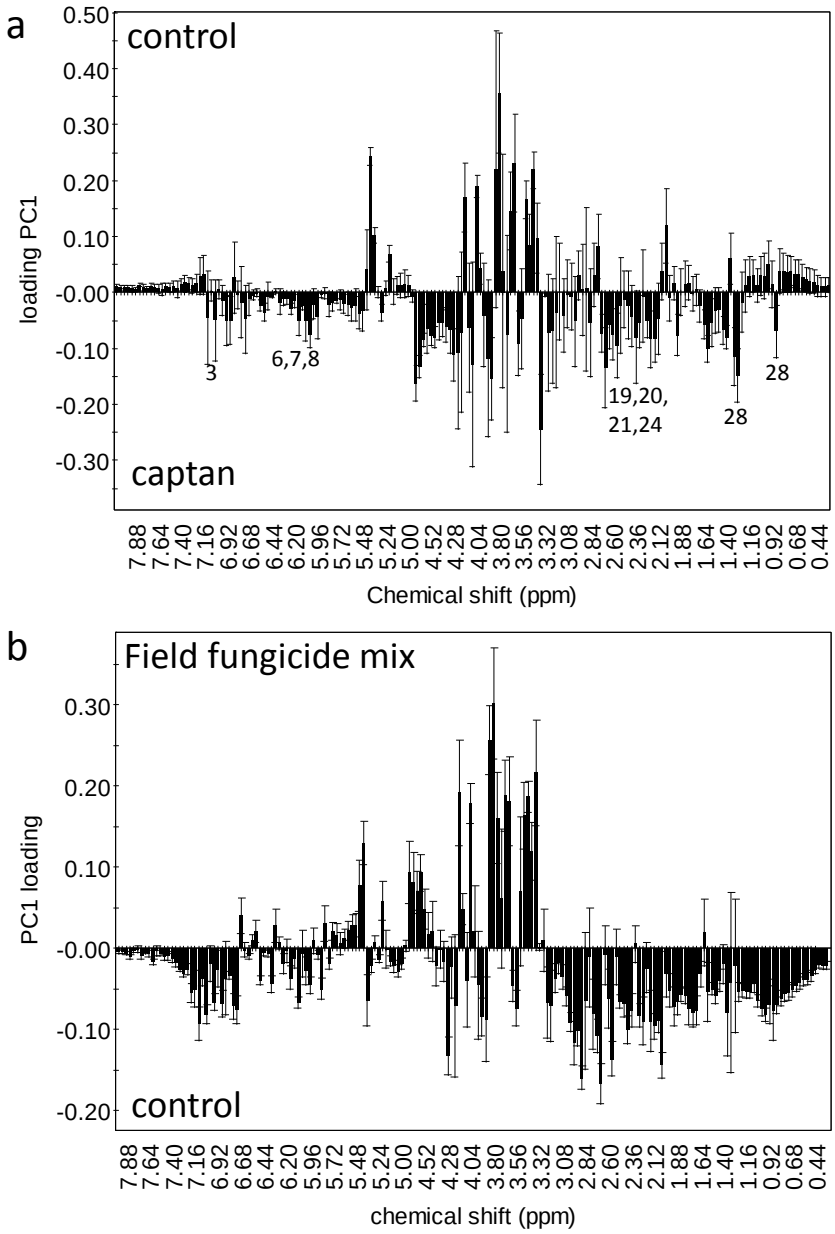

Figure 6. (a) Loading column plot of PCA of control versus captan, (b) loading column plot of PCA of control versus field fungicide mix.

Relative quantification of selected metabolites

The NMR bucket table was submitted to ANOVA, to compare the average signal intensities in each variable bucket and to confirm whether differences seen in the multivariate data analysis were statistically significant. The results confirmed what was found in the PCA, and some examples of buckets with significantly different average intensities are shown in Figure 6. The buckets containing tyrosine and haemanthamine signals (Figure 7a,b) for example, were higher in the before treatments. The treatments containing formaldehyde alone or in a mixture had the highest average levels. Signals belonging to sucrose were significantly higher in the samples treated after planting, as is shown in Figure 7c.

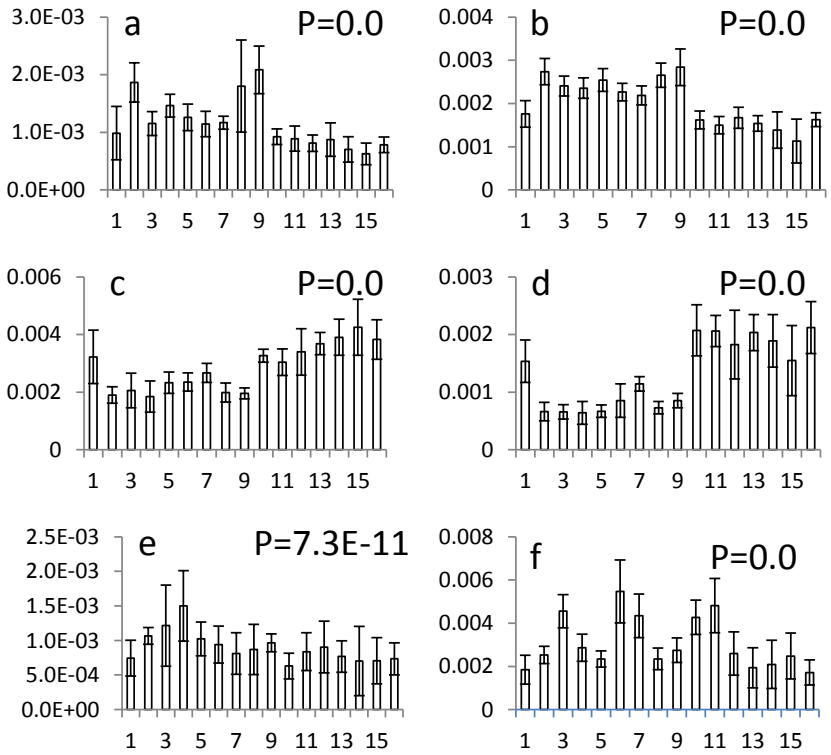

Figure 7. Relative quantification of selected compounds based on peak areas of associated signals (mean ± SD, n=18 for treatment 1, n=9 for other treatments). Chemical shifts were (a) 7.20: tyrosine, (b) 5.96: haemanthamine, (c) 5.44: sucrose, (d) 5.20: glucose, maltose, (e) 3.12: ethanolamine, (f) 2.60: citric acid. The P-values of the ANOVA between the different fungicide treatments are shown in the Figure.

The bucket of δ 5.20 contained signals of glucose and maltose. On closer inspection of this chemical shift region in all the samples, the relative ratio of the two doublet signals were seen to be different based on the timing of fungicide application. The maltose doublet signal intensity stayed constant between treatments. The glucose doublet signal at δ 5.19 was approximately double the size of the maltose doublet signal at δ 5.17 in the control. In samples treated before planting, the doublet signals were about equally intense. In contrast, samples treated after planting also had more intense glucose doublet signals, but with more than double the intensity of the doublet at δ 5.17. This is shown in Figure 8, where these signals of representative samples from the different times of application are compared.

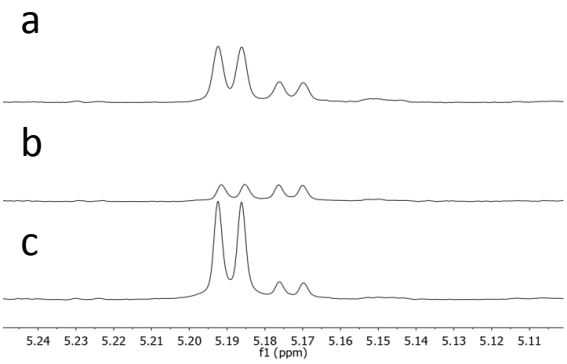

Figure 8. The doublet signals of glucose (δ 5.19) and maltose (δ 5.17) in a representative ^{1}H NMR spectrum of (a) control bulbs (treatment 1), (b) bulb treated with captan (treatment 3), (c) bulb treated with fluazinam after planting (treatment 10).

Discussion

In conventional agricultural systems, applied fungicides may affect the metabolism of the crop plant. This can have important implications for plants cultivated for the production of medicinal compounds. ^{1}H NMR was used to analyze the fungicide-treated *N. pseudonarcissus* cv. Carlton bulbs, which allowed the detection and quantitative analysis of major primary and secondary metabolites. A targeted analysis of galanthamine in the bulb samples revealed differences in the concentration between certain treatments. The average galanthamine concentration was as low as half that of the control bulbs in some treatments (Figure 1). Analysis of the entire ^{1}H NMR spectrum by PCA revealed grouping based on the comparison of all the variables between samples. The PCA score plot revealed that the time of application of fungicides was an important factor in explaining the variance in the dataset. The bulbs treated with

fungicides before planting generally had more intense signals in the aromatic region of the ^{1}H NMR spectrum. Galanthamine, haemanthamine, narciclasine and the alkaloid precursors tyrosine and 4-hydroxyphenylpyruvate were important for the discrimination. While these signals were also present in the other samples, they were less intense in the control samples and those treated in the field. Similarly, major sugar signals were seen in all the samples, but those belonging to sucrose were higher in bulbs treated in the field. Changes in carbohydrate metabolism were also seen by the altered ratios of glucose relative to maltose in different treatments compared to the control.

The samples treated with the sterol biosynthesis inhibitor (SBI) prochloraz (treatment 4), had a lower average galanthamine concentration and higher ethanolamine levels than the control. Increased ethanolamine suggests an effect on the metabolism of cell membrane components (Rontein et al., 2003). Two metabolite signals (1-*O*-ethyl-β-glucoside and fatty acid) were also seen only in this fungicide treatment. The contact fungicide captan (treatment 3) works on multiple biochemical targets to affect fungal pathogens. Bulbs treated with this fungicide also had a lower average galanthamine concentration. This is in spite of having more intense signals in the alkaloid and aromatic compound region, as for the other samples treated before planting. A shift in the bulb metabolism was caused by this fungicide treatment, as seen by altered levels of amino acids and organic acids, in particular citric acid.

The field fungicide treatments that had lower galanthamine concentrations were characterized by increased unsaturated fatty acids, as well as increases in certain organic acids. Changes in sugars were also seen as compared to the controls. These differences as well as the lower galanthamine levels may all be a reflection of the effect of the fungicides on metabolism of the photosynthetic tissue in the field. Bulbs are storage organs and much of the products of photosynthesis and other metabolites are transported down to them when the aboveground parts senesce (Hanks, 2002). The effect of foliar fungicide treatments can still be seen in the metabolite profiles of the bulbs after leaf senescence.

A study by Kucht et al. (2004) describes a decrease of alkaloids as a result of fungicide application in *Ipomoea*. This was ascribed to the inhibitory effect of the fungicide on an endophytic fungus, which was hypothesized to be involved in alkaloids' production. One could speculate that the foliar fungicide applications altered the amount of galanthamine and other alkaloids as a result of their effects on an associated fungus. *Narcissus* roots are known to be associated with mycorrhizal fungi (Chilvers and Daft, 1981), however the occurrence of any leaf endophytes is not known. Changes in metabolite patterns in the fungicide-dipped bulbs may also be due to a direct or indirect (via the plant) effect on symbiotic microorganisms (Diedhiou et al., 2004). Studies on

the effects of different fungicides on mycorrhizal symbioses showed mixed results, depending on the agent applied (Diedhiou et al., 2004). A study on the effects of two different SBIs on carrot roots showed one to alter root sterol composition and have a toxic effect on the symbiosis, while the other had minimal impact on plant sterols and fungal colonization (Campagnac et al., 2008). The authors emphasized the importance of studying the impact of fungicides on the functioning of symbiotic interactions in crops. The decreased galanthamine in plants with certain fungicide applications as compared to the control could suggest such indirect effects. However, such interactions are difficult to demonstrate clearly in a field study.

The approach used in this study was able to address the question of whether fungicide applications in *N. pseudonarcissus* cv. Carlton cultivation affect the galanthamine concentration in the bulbs. For most of the treatments the galanthamine concentrations were similar. The treatments with lower levels were all with individual fungicides applied either before or after planting. In the large-scale cultivation of *N. pseudonarcissus* cv. Carlton mixtures of fungicides are typically used. In this study the treatments that received a mixture before planting (treatment 8), and a mixture before planting as well as in the field (treatment 9) in fact had the highest average galanthamine concentrations. This was in spite of the mixtures containing one or more of the fungicides that had lower galanthamine in the individual treatments. The standard cultivation practices for *N. pseudonarcissus* cv. Carlton in terms of fungicide treatment thus seem suitable for the cultivation of the plant as raw material for galanthamine extraction, at least in terms of its effect on the compound of interest. In the cultivation of medicinal plants for secondary metabolites the potential effect of pesticides and other agrochemicals should be taken into account.

Chapter 7. Toxic residues in *Narcissus* bulbs

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Abstract

The production of medicinal crops is usually done under a Good Agricultural Practice (GAP) scheme, with each step of the process carefully documented and tracked. The presence of toxic residues accumulated during cultivation is unwanted in the final plant material. The ornamental sector of agriculture in the Netherlands involves high-intensity farming, with high input of pesticides. For ornamental crops not intended for human consumption no laws exist for limits of pesticide residue levels. Like other ornamental bulbs no Maximum Residue Limits (MRLs) are defined for *Narcissus* cut flowers or bulbs. In the very strictly regulated pharmaceutical industry every impurity in the process of production needs to be characterized and proven to be safe. It is therefore important to know whether there are pesticide residues in the raw starting material to be used for medicinal purpose. Similarly the presence of mycotoxins may be an issue when cultivating *Narcissus* as pharmaceutical raw material. Some of the fungi that infect *Narcissus* bulbs in the Netherlands belong to genera that are known to produce harmful mycotoxins. The aim of this study was to determine if traces of the pesticides applied during the growing season can be detected after harvest of *Narcissus* bulbs. A field study was conducted with various pesticide treatments, and LC-MS and GC-MS was used to determine the levels of residues where possible. *Narcissus* bulbs samples heavily infected with *Fusarium oxysporum* were analyzed to determine whether any mycotoxins were present in the infected tissue.

Determination of pesticide residues in *Narcissus* bulbs

Introduction

Narcissus (daffodil) is one of the main ornamental flower crops in the Netherlands. The ornamental sector of agriculture in the Netherlands is characterized by high-intensity farming, with high input of fertilizers and pesticides (Ten Berge, 2000). In recent years, increased awareness of the detrimental effects of pesticides on the environment and potential risk to human health has resulted in policy and legislation to lower the use of these agrochemicals (Wossink and Feitshans, 2000; Zuin and Vilegas, 2000). This has been taken very seriously in the food sector in Europe, with laws specifying Maximum Residue Limits (MRLs). MRLs differ between food types and also between pesticides, and is a value calculated based on the risk of the particular compound to human health and how the compound accumulates in or on the food. Typical limits are in the range of 0.01-3.00 mg/kg (Titato et al., 2007).

For ornamental crops not intended for human consumption no regulations exist for limits of pesticide residue levels. Therefore, no Maximum Residue Levels (MRLs) are defined for *Narcissus* cut flowers or bulbs. In recent years, interest has been growing to cultivate *Narcissus* as a medicinal plant crop. This comes after the discovery that the alkaloid galanthamine is produced by the plant, and accumulates in particular in the bulb (Heinrich and Teoh, 2004). The cultivation of medicinal crops is quite different from ornamental crops. The production of medicinal crops is usually done under a Good Agricultural Practice (GAP) scheme, with each step of the process carefully documented and tracked. The European Pharmacopoeia set some pesticide MRLs for medicinal crops. Monographs are being added that include MRLs for medicinal plant material, but for many crops they do not exist yet. The presence of pesticide residues is unwanted in any crop to be used as a medicine. Even if a compound is to be isolated from the material, the extraction method may concentrate unwanted pesticide traces. In the very strictly regulated pharmaceutical industry every impurity in the process of production needs to be characterized and proven to be safe. It is therefore important to know whether there are pesticide residues in the raw starting material.

Narcissus bulbs are already available in large quantities, and the production methods are well established. Converting this ornamental crop into a pharmaceutical crop may involve adjustment of some cultivation practices. An assessment is needed of how the current agricultural production methods affect the quality of the bulbs in terms of galanthamine levels and unwanted chemical residues. This will give an indication of how agricultural practices need to be adapted to form part of a GAP scheme for the production of galanthamine for the pharmaceutical industry. The aim of this study was

to determine if traces of the pesticides applied during the growing season can be detected after harvest of *Narcissus* bulbs. A selection of pesticides typically used in *Narcissus* cultivation the Netherlands was chosen for this study (Table 1).

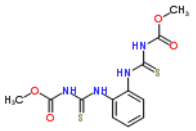
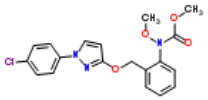
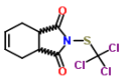
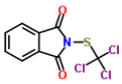
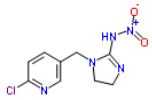
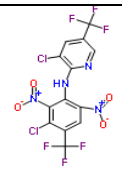
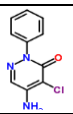
The determination of pesticide residues in plant material usually involves extraction with an organic solvent, cleanup and/or concentration of the extract, and identification and quantification of the residues. The ideal method should be selective so that as much as possible of the pesticides in question are extracted, and as little as possible other non-pesticide matrix components are co-extracted. The analysis method should be sensitive so that compounds present in very low concentrations can be detected, and unambiguously identified (Alder et al., 2006; European Commission Health and Consumers (SANCO), 2007).

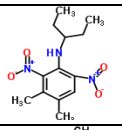
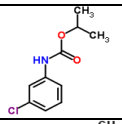
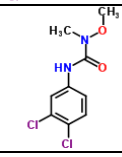
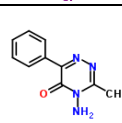
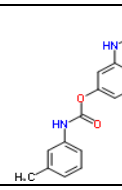
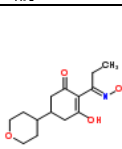
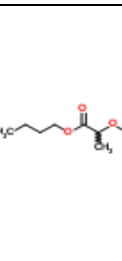
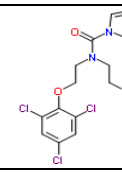
Most modern pesticide determination methods aim to detect multiple compounds at the same time. The challenge in developing such a multi-residue method is to achieve satisfactory extraction of many compounds with diverse structures. Acetone, ethylacetate or acetonitrile are most often used as extraction solvents for that purpose (Luke et al., 1975; Andersson and Pålsheden, 1991; Anastassiades et al., 2003; Mol et al., 2007). The plant matrix being extracted and the solvent used will largely determine how clean the extract is. Many pesticide detection methods require one or more cleanup steps. These involve filtration, liquid partitioning, and/or water removal using anhydrous salts. The use of solid phase extraction (SPE) and microextraction (SPME), and matrix solid-phase dispersion (MSPD) is increasingly popular as a variety of convenient commercial products become available (Wang et al., 2007).

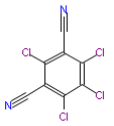
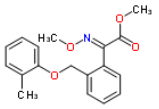
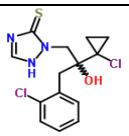
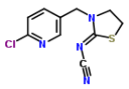
Gas chromatography (GC) in combination with electron capture, nitrogen-phosphorous, and/or flame photometric detectors has traditionally been the most common technique for pesticide analysis. GC gives good resolution and separation, allowing many compounds to be analyzed in one run. However, not all compounds are volatile and suitable for GC without derivitization. Liquid chromatography (LC) was not commonly used for pesticide analysis in the past. This was mainly due to detectors (UV and fluorescence detectors) not being selective or sensitive enough. Newly developed pesticides tend to be more polar and thermally labile, so that liquid chromatography (LC) is a more suitable option (Kruve et al., 2008). Advances in mass spectrometric detectors have made LCMS systems very popular for pesticide analysis. For both LC and GC, the advantage of using an MS-detector is that identification and quantification can be performed at the same time (Alder et al., 2006). The ability to select specific ions for monitoring in selective ion monitoring (SIM) mode increases sensitivity, while the use of tandem MS (MS/MS) allows certain identification to eliminate false positive

results in pesticide screenings. GC-MS (and GC-MS/MS) and LC-MS/MS are often used as complementary techniques to analyze a variety of compounds.

Table 1. Summary of the pesticides and herbicides in this study.

Name	**Pesticide class**	**Molecular weight**	**Elemental composition**	**Chemical structure**
Thiophanate methyl	Benzamidazole fungicide	342.4	$C_{12}H_{14}N_4O_4S_2$	
Pyraclostrobin	Carbanilate/strobi-lurin fungicide	387.8	$C_{19}H_{18}ClN_3O_4$	
Captan	Pthalimide fungicide	300.6	$C_9H_8Cl_3NO_2S$	
Folpet	Pthalimide fungicide	296.5	$C_9H_4Cl_3NO_2S$	
Imidacloprid	Chloronicotinoid insecticide	255.7	$C_9H_{10}ClN_5O_2$	
Fluazinam	Pyridine fungicide	465.1	$C_{13}H_4Cl_2F_6N_4O_4$	
Chloridazon	Pyridazinone herbicide	221.6	$C_{10}H_8ClN_3O$	

Pendimethalin	Dinitroaniline herbicide	213.6	$C_{13}H_{19}N_3O_4$	
Chlorprofam	carbinilate herbicide	213.6	$C_{10}H_{12}ClNO_2$	
Linuron	substituted urea herbicide	249.1	$C_9H_{10}Cl_2N_2O_2$	
Metamitron	triazinone herbicide	202.2	$C_{10}H_{10}N_4O$	
Phenmedipham	Carbinilate herbicide	300.3	$C_{16}\ H_{16}N_2O_4$	
Tepraloxydim	Cyclohexene oxime herbicides	341.8	$C_{17}H_{24}ClNO_4$	
Fluazifop-P-butyl	Aryloxyphenoxy-propionic herbicide	383.4	$C_{19}H_20F_3NO_4$	
Prochloraz	Amide fungicide	376.67	$C_{15}H_{16}Cl_3N_3O_2$	

Chlorothalonil	Organochlorine fungicide	265.9	$C_{12}H_{13}N_3$	
Kresoxim-methyl	Strobilurin fungicide	313.8	$C_{18}H_{19}NO_4$	
Prothioconazole	Conazole fungicide fungicide	344.2	$C_{14}H_{15}Cl_2N_3OS$	
Thiacloprid	Chloronicotinoid insecticide	252.73	$C_{10}H_9ClN_4S$	

Materials and Methods

Cultivation and experimental setup

Bulbs of *Narcissus pseudonarcissus* cv. Carlton were planted in Lisse, The Netherlands, in November 2008. For each agrochemical treatment, a plot consisting of four rows of bulbs (two rows of 10 bulbs and two rows of 11= total 42 bulbs) was planted. All treatments received a standard fertilizer treatment of nitrogen and potassium. The standard nitrogen application was 110 kg/hectare of Kalksalpeter (Ca(NO3)2)with 19% Ca and 15.5% N, consisting of 14.4% N-NO3, 1.1% N-NH_4) applied, applied in three applications of 40 kg, 40 kg and 30 kg. The standard potassium treatment consisted of 150 kg of Patentkali (K_2SO_4 and $MgSO_4$, 30% K as K_2O, 10% Mg as MgO and 42% S as SO_3) given in two applications of 75 kg each. Other agrochemicals were applied according to the scheme presented in Table 2 and 3. One plot received no pesticide application and was used as a control. Bulbs were lifted in July 2009, and dried for two weeks at 20 °C according to standard ornamental cultivation methods.

Sample preparation

To prepare bulbs for analysis, individual bulbs were cleaned to remove soil, dead outer scales and roots. Each bulb was frozen in liquid nitrogen and ground to a fine powder

using a Waring laboratory blender. The ground bulb material was freeze-dried and kept at -20 °C until analysis. For sample preparation, 3 g of freeze-dried plant material was weighed into 50 ml Corning centrifuge tubes and distilled water was added (6 g). The tubes were vortexed for 1 min to rehydrate the material to 66% moisture. Acetone (10 ml) was added to each tube and vortexed for 30 seconds. After adding 10 ml dichloromethane and 10 ml petroleum ether tubes were vortexed again, and then centrifuged at 35000 rpm for 10 minutes. After centrifugation 10 ml of the supernatant was collected in 15 ml glass tubes.

Table 2. Summary of treatments applied in water bath before planting.

treatment	**agrochemical applied**	**active substance**	**Amount in water bath (% w/v)**
1	none	none	none
2	Topsin	thiophante methyl	0.50
3	Securo	Pyraclostrobin folpet	1.50
4	Luxan Securo	Captan folpet	0.5 1.50
5	Admire	Imidacloprid	0.04
6	Shirlan	Fluazinam	0.25

Table 3. Summary of agrochemical application in the field.

treatment	**Agrochemical applied**	**Active substance**	**Amount applied (per ha)**
7	Pyramin	Chloridazon	3 kg
8	Stomp	Pendimethalin	2 L
	Linuron	Linuron	1.5 L
	ChlIPC	Chlorpropham	4 L
9	Goltix	Metamitron	0.5 L
10	Herbasan	Phenmedipham	2 L
11	Aramo	Tepraloxydim	2 L
12	Fusilade	Fluazifop-P-butyl	1.5 L
13	Allure	Prochloraz	2 L
14	Kenbyo	Kresoxim-methyl Mancozeb	0.8 L
15	Chlorthalonil	Chlorthalonil	2 L
16	Rudis	Prothioconazole	0.4 L
17	Calypso	Thiacloprid	0.25 L

For LCMS analysis, the supernatant was evaporated to dryness under a stream of nitrogen. Once dry, the extract was reconstituted in 1 ml methanol and filtered through a 0.45 µm PTFE syringe fliter (VWR, Leicestershire, UK) before injection. For GC-MS analysis, the supernatant was concentrated to 1 ml under a stream of nitrogen gas. Solid phase extraction (SPE) was carried out using 3 ml cartridges containing 200 mg Primary Secondary Amine (PSA) Bonded silica (Supelco, Bellafonte, PA). Anhydrous magnesium sulphate was added to the top of the SPE sorbent to remove any residual water still present in the sample. Cartridges were mounted on a Chromabond® vacuum manifold (Machery-Nagel, Düren, Germany) and conditioned with 5 ml acetone. After applying the samples, cartridges were eluted with 10 ml acetone. The eluent was concentrated to 1 ml and placed in dark vials for injection.

Preparation of matrix-matched standards

Individual stock solutions of the pesticides at 1 mg/ml were prepared in methanol and stored at -20 °C when not in use. Mixed working solutions containing 0.001 mg/ml of each pesticide were prepared for the LC- and GC-amenable pesticides by dilution of the stock solution in methanol and acetone, respectively. For the LC-amenable compounds, serial dilutions of the working solution were made in methanol to give five solutions of 0.015-0.3 µg/ml. Control bulbs were extracted as described above to produce blank matrix extracts. The dried extracts were redissolved in the standard solutions to give a working range of 0.005-0.1 mg/kg. For the GC-amenable compounds, serial dilutions were made of the working solution in acetone to give five concentrations from 0.003-0.75 mg/ml. Control bulbs were extracted as described above and dried completely after SPE cleanup. The dried extracts were redissolved in the standard solutions to give a working range of 0.001-0.25 mg/kg.

LC-MS determination

An Agilent 1100 Series LC/MSD system was used for determination of LC-MS-amenable compounds. An APCI probe was used in positive mode. The MSD spray chamber gas temperature and vaporizer temperature were both set at 350°C. Drying gas flow was 4.0 mL/min, and nebulizer pressure was 40 psig. Capillary voltage for positive mode was 4000V, and corona current was 4.0 µA. Liquid chromatographic separation was performed on a reversed phase Phenomenex column (Luna 5 micron C18 100A, 150 x 4.60 mm). The mobile phase consisted of LCMS grade Water (A) and Methanol (B) (LCMS Chromasolv, Fluka Analytical/Sigma, Steinheim, Germany) each containing 0.1% formic acid (Fluka). The solvent composition of the mobile phase started with 20% B for 3 min, increasing to 90% B over 5 min, holding at 90% B for 11 min and then returning to 20% B during 1 min. The total run time was 20 minutes.

GC-MS Determination

Gas Chromatographic analysis was carried out on an Agilent 7890A GC system with a 5975C quadropole MS detector and 7693 autosampler. The system was fitted with a DB-5 column (30 m x 250 µm x 0.25 µm). Injection was done in split mode (1:10) at 270°C. The oven temperature was held at 80°C for 1 min, increased to 150°C at 25°C/min, increased to 230°C at 10°C/min, then increased to 250°C at 2.5°C/min, then increased to 280°C at 10°C/min, and finally held at 280°C for 15.2 min (total run time was 38 minutes). Helium was used as carrier gas at a flow rate of 1.2 ml/min. For analysis in scan mode, ions from 50-500 m/z were detected. The time-programmed SIM mode was set up in nine groups as follows: 3.0-10.0 min, 10.0-11.5 min, 11.5-12.5 min, 12.5-13.15 min, 13.5-14.0 min, 14.0-14.95 min, 14.95- 16.0 min, 16.0-17.34 min, 17.34-38.0 min with the ions monitored in each group indicated in Table 4. In all cases the base peak was used for quantitation, except in the case of chloridazon the ion 221 was used.

LC-MS method validation

Recovery of the extraction method was assessed by preparing six samples from control bulbs (not treated with any pesticides) in the standard way. Three of the samples were spiked with a solution of pesticide standards (to give equivalent of 0.01 mg/kg of each compound) before extraction. Three of the samples were extracted, dried and reconstituted in pesticide standard solution in methanol to give an equivalent of 0.01 mg/kg of each pesticide. The average peak area of each pesticide added before extraction was compared to that of each pesticide added after extraction to determine the percentage recovery. Reproducibility of the method was assessed by injecting the same sample (blank matrix spiked with pesticide standard solution to give equivalent of 0.01 mg/kg of each compound) three times on one day, and calculating the percentage standard deviation of the average peak areas for each compound. To assess the linear range of the method, matrix-matched standard solutions were prepared of the pesticides. Five concentrations from 0.005-0.1 mg/kg were prepared in triplicate for each compound. A standard curve was prepared by plotting the concentrations against average area of the pesticide peaks. Linear range was estimated as the range of concentrations over which the r^2>0.99.

GC-MS method validation

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Results

LC-MS

A mixture of the LC-amenable pesticide standards was used to develop a mobile phase system that separated the compounds (Figure 1). Using the mass detector, compounds were inspected in scan mode (100-600 m/z) to determine their ionization patterns. For most compounds the molecular ion or M+1 ion was the most abundant and was chosen for quantitation in SIM mode. An exception was thiophanate-methyl, whose most abundant ion was 151 ($[M+H-CH_3-OCONH-CH_3OCONHCS]^+$). The chosen ions were used to create a time-programmed SIM method, monitoring the chosen ions in time windows when these compounds eluted (Table 4). Flow injection analysis (FIA) of individual pesticides was carried out to determine optimal fragmentor voltage for SIM analysis of the chosen ions. A fragmentor voltage of 90 V was used for analysis as it gave good abundance of all the quantitative ions.

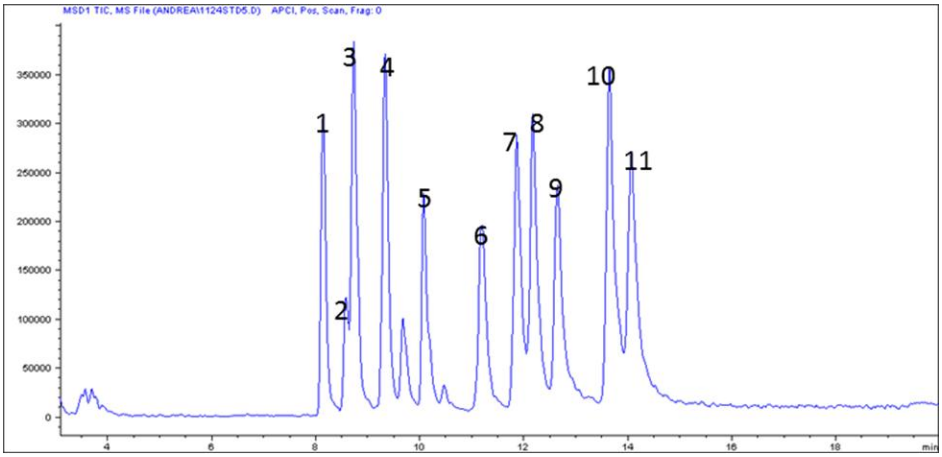

Figure 1. Scan mode LCMS APCI chromatogram of a standard solution of pesticides. Peak identification: 1 imidacloprid, 2 metamitron, 3 thiacloprid, 4 thiophanate-methyl, 5 phenmedipham, 6 tepraloxydim, 7 prochloraz, 8 pyraclostrobin, 9 prothioconazole, 10 fluazinam, 11 cycloxidim.

The extraction method used was based on methods by Schenck et al., 2002 and described in RIVM Report number 638817014 (RIVM, 1996). Recovery was reasonable for most of the compounds, with all except cycloxidum being greater than 70%. To assess the reproducibility and linearity of this method, it was necessary to work with matrix-matched samples. In mass spectrometic methods a "matrix-effect", a change in the ionization efficiency in the presence of other compounds, is often observed (Kebarle and Tang, 1993). For accurate quantitative work it is recommended to prepare standard solutions in a blank sample extract, so that this may be is taken into account (SANCO, 2004). For this reason quantitation was done using matrix-matched standards. The result of injecting the same sample three times showed average peaks with less than 5% standard deviation (Table 5). The fluazinam peak was less reproducible and had an average standard deviation of 13%.

With the compounds in the background of the sample matrix, two of the compounds, prothioconazole and cycloxidim, showed ion suppression (i.e. the ion signal was much less intense that in the pure solution). For this reason when trying to determine linear range only two points could be obtained for the curve for the higher concentration points. For these two compounds it was not possible to determine linear range, and only a rough presence/absence screening could be done. All the other compounds were linear over a range suitable for pesticide residue determination (Table 5).

Samples of bulbs treated with the LCMS-amenable pesticides were analyzed using the developed method to see if traces of the active compounds could be found. The only compound detected within the linear range of the method was pyraclostrobin (Table 8). Imidacloprid, thiophanate-methyl, phenmedipham, tepraloxydim and prochloraz were present in trace amounts that could not be quantitifed accurately as they were out of the range of quantitation (below Limit of Quantiation). Metamitron, fluazinam and prochloraz were not detected at all. Cycloxidum and prothioconazole were not determined due to severe ion suppression of the matrix-matched standards.

Table 4. Time program and ion fragmentation patterns of LCMS-amenable pesticides. SIM monitoring was performed using the ion marked with an asterisk (*).

Group	**Time window**	**compound**	**MW**	**RT**	**Ions (% Relative abundance)**
1	5.0-10.10 min	Imidacloprid	255.7	9.25	256*
					212
		Metamitron	202.2	9.68	203.1 (100)*
		Thiacloprid	252.7	9.88	253.2 (100)*
					285.3 (10)
2	10.10-10.90 min	Thiophanate methyl	342.4	10.504	343 (58)*
					151 (100)
					134 (98)
3	10.90-12.6 min	Phenmedipham	300.3	11.15	333.3 (100)*
					334.2 (18)
					318.1 (5)
		Tepraloxydim	341.8	11.76	342.3 (100)*
					250.2 (75)
					282.3 (25)
		Prochloraz	375.6	11.97	376 (100)*
					340 (58)
					284 (47)
		Pyraclostrobin	387.8	12.08	388 (100)*
					356 (50)
					390 (34)
		Prothioconazole	344.2	12.28	344.1 (100)*
					346.2 (70)
					308.1 (10)
		Fluazinam	463.0	12.661	384*
4	12.6-20.0 min	cycloxidim	325.4	13.05	326.4 (100)*
					280.2 (50)
					312.3 (10)

Table 5. Recovery, reproducibility, linear range test for LCMS-amenable pesticides:

Pesticide	Average Recovery (%, n=3)	Reproducibility (% RSD)	Linear range (mg/kg) (R^2)	Limit of Detection (mg/kg)
Imidacloprid	71.2		0.005-0.1	0.005
		5.21	(0.995)	
Metamitron	79.7		0.005-0.1	0.001
		5.56	(0.997)	
Thiacloprid	84.4		0.005-0.1	0.001
		4.67	(0.996)	
Thiophanate	81.2		0.025-0.1	0.010
methyl		4.76	(0.998)	
Phenmedipham	93.5		0.005-0.1	0.001
		6.83	(0.995)	
Tepraloxydim	92.87		0.01-0.1	0.005
		2.63	(0.998)	
Prochloraz	73.6		0.005-0.1	0.001
		3.06	(0.998)	
Pyraclostrobin	71.4		0.005-0.1	0.001
		1.74	(0.996)	
Prothioconazole	75.3	2.00	-	0.050
Fluazinam	82.0		0.005-0.1	0.005
		13.00	(0.993)	
Cycloxidum	68.7	3.80	-	0.050

GC-MS

An oven temperature program was developed to achieve separation of all the GC-amenable compounds (Figure 2). A mixture of pesticides was injected in the GC-MS and monitored in scan mode (100-600 m/z). The fragmentation pattern of each pesticide was examined to select quantitative ions for SIM mode. The base peak (most abundant ion) was used as quantitative ion in all cases except for chloridazon. In this case the M^+ ion was chosen as quantitation ion, as it had a high enough abundance and was not an ion fragment common to many molecules (the base peak was 77 m/z, which is a very common ion of compounds with aromatic rings). In the case of captan and folpet, due to their very similar structures the most abundant ion was 79 m/z in both cases. For each

compound, two additional ions were monitored to enable confirmation of identity. Using the quantitation and identifier ions, a time-programmed SIM program was developed for analysis (Table 6).

Table 6. Time program and ion fragmentation patterns of GCMS-amenable pesticides. SIM monitoring was performed using the ion marked with an asterisk (*).

Group	Time window	compound	MW	RT	Ions (% abundance)
1	3.0-10.0	Chlorprofam	213.6	9.04	153 (100)*
					127 (48)
					213 (14)
2	10.0-11.5	Chlorothalonil	265.9	10.80	265.9 (100)*
					263.9 (81)
					228.9 (10)
3	11.5-12.5	Linuron	249.1	12.01	61.1 (100)*
					186.9 (69)
					124.0 (40)
4	12.5-13.15	Pendimethalin	281.3	13.02	252.1 (100)*
					207.5 (49)
					281.0 (19)
5	13.5-14.0	Captan	300.6	13.27	79.0 (100)*
					113.9 (19)
					206.9 (9)
		Folpet	296.5	13.42	79.0 (100)*
					104.0 (88)
					147.0 (71)
6	14.0-14.95	Kresoxim-methyl	313.3	14.803	116.0 (100)*
					206.0 (36)
					131.0 (22)
7	14.95-16.0	Fluazifop-P-butyl	383.4	15.07	282.0 (100)*
					254.0 (50)
					383.0 (42)
8	16.0-17.34	Chloridazon	221.6	16.84	221.0 (67)*
					105.0 (23)
					207.0 (11)
					77.0 (100)

The extraction method used was based on methods by Schenck et al., 2002 and described in RIVM Report number 638817014 (RIVM, 1996). Cleanup of extracts using primary secondary amine (PSA) solid phase extraction (SPE) cartridges was performed to provide cleaner samples with less matrix co-extractants. Recovery of the extraction method was assessed and found to be poor for most of the compounds (between 30-40% for most compounds). Two of the more volatile compounds (chlorothalonil and pendimethalin) were lost completely in the drying and SPE steps. Captan had a recovery of more than 100%. A comparison of the SIM chromatogram of a blank matrix and one spiked with a mixture of pesticides (Figure 3) shows that in the region where captan and folpet elute a matrix component that was not removed by the cleanup is present. This likely caused an enhancing matrix effect and caused the peak to be overestimated. In an attempt to improve the recovery of these compounds, a recovery experiment was performed where the cleanup step was omitted. The samples prepared in this way were too dirty to inject in the GCMS, as solid precipitant was present which could clog the needle and shorten the lifetime of the capillary column. The drying steps likely also contributed to the poor recovery, but they were necessary to remove the chlorinated solvent and concentrated the sample to enable detection within limits of the equipment's sensitivity.

Matrix-matched standard solutions of each pesticide were injected three times on one day to evaluate the reproducibility of the method. Table 7 shows the average relative standard deviations for each compound, which were quite low for most compounds. The highest standard deviation was for captan, which is known to give problems with reproducible ionization (Pihlström et al., 2007). Most of the compounds showed a good linear response (r^2>0.99) over a range of 0.005-0.25 mg/kg, while chlorprofam and chlorothalonil showed a linear response down to 0.001 mg/kg.

Samples of bulbs treated with the GCMS-amenable pesticides were analyzed using the developed method to see if traces of the active compounds could be found. Apart from samples treated with captan and folpet, no traces of the pesticides could be found in the bulb samples (Table 8). It may be that due to the poor recovery of the method they are present but below the limit of detection. The results indicate the presence of captan and folpet at high levels in the bulbs. However, due to the excessively high recovery seen in Table 7 caused by an interfering peak, these results are likely to be overestimated. The level of captan shown in Table 8 is out of the linear range of quantitation determined in this study, so cannot be considered accurate. The level of folpet is at the upper range of linearity for this study.

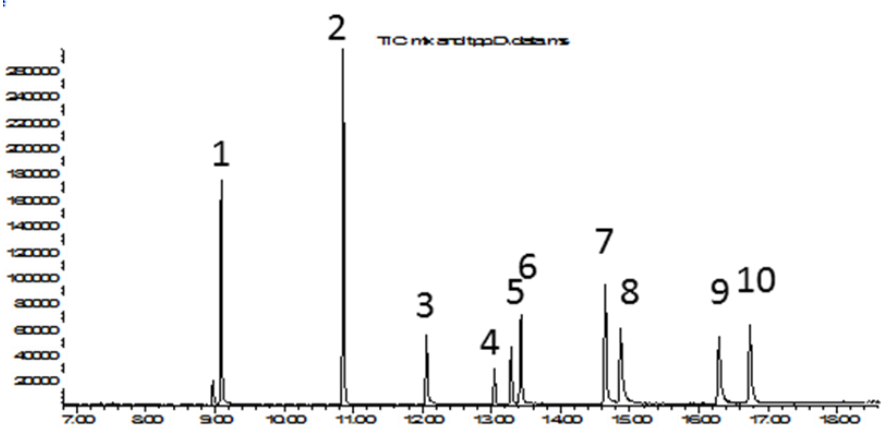

Figure 2. Scan mode GCMS chromatogram of a standard solution of pesticides. Peak identification: 1 chlorprofam, 2 chlorothalonil, 3 linuron, 4 pendimethalin, 5 captan, 6 folpet, 7 kresoxim-methyl, 8 fluazifop-P-butyl, 9 chloridazon, 10 Triphenyl phosphate (IS).

Table 7. Recovery, reproducibility, linear range test for GCMS-amenable pesticides:

Pesticide	Average Recovery (%, n=3)	Reproducibility (% RSD)	Linear range (mg/kg) (r^2)	Limit of detection (mg/kg)
Chlorprofam	36.3	4.4	0.001-0.25 (0.999)	0.001
Chlorothalonil	0.0	5.8	0.001-0.25 (0.997)	0.001
Linuron	40.4	5.0	0.005-0.25 (0.997)	0.005
Pendimethalin	0.0	3.9	0.005-0.25 (0.999)	0.005
Captan	147.2	8.1	0.005-0.25 (0.997)	0.005
Folpet	28.0	4.3	0.005-0.25 (0.998)	0.005
Kresoxim-methyl	31.7	5.6	0.005-0.25 (0.999)	0.005
Fluazifop-P-butyl	29.9	4.1	0.005-0.25 (0.999)	0.005
Chloridazon	60.3	6.2	0.005-0.25 (0.995)	0.005

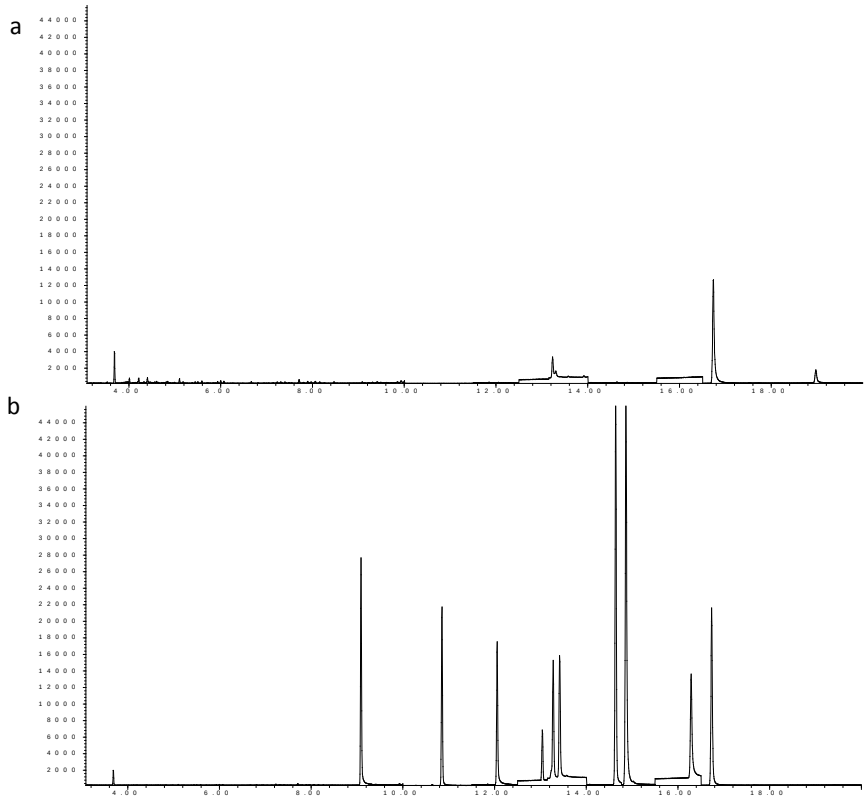

Figure 3. Selective ion monitoring mode GC-MS chromatograms of (a) blank bulb matrix and (b) bulb matrix spiked with pesticide mixture.

Discussion

To develop a method suitable for all the pesticides to be analyzed, acetone was chosen as an extraction solvent. The solubility of all the compounds to be analyzed in acetone, as well as its availability, price and low toxicity made this a good choice. A method was developed based on those described in Schenck et al. (2002) and the National Institute of Public Health and the Environment of the Netherlands (RIVM) Report 638817014 (RIVM 1996). In these methods the plant material is extracted, and the extracts concentrated to allow detection of low levels of pesticide residues. In other methods, for example the QuEChERS method described by Anastassiades and co-workers (2003), the extracts are not concentrated, but analysis is done using LC and GC equipment with MS/MS capabilities with much greater sensitivity. In this study the availability of single

quadrupole LCMS and GCMS only made it necessary to use a method that would include a concentration step. In terms of linearity and reproducibility the LCMS and GCMS based methods were good. The recoveries were good for the LC-amenable compounds, but poor for the GC-amenable compounds. A significant proportion of the compounds were lost in the cleanup and concentration steps. However, as described previously, these steps could not be avoided.

Table 8. Results of sample analysis LC-MS- and GC-MS-amenable pesticides.

LCMS Pesticide	**Sample mean (mg/kg) ± RSD**	**GCMS Pesticide**	**Sample mean (mg/kg) ± RSD**
Imidacloprid	below LOQ	Chlorprofam	ND
Metamitron	ND	Chlorothalonil	ND
Thiacloprid	ND	Linuron	ND
Thiophanate methyl	below LOQ	Pendimethalin	ND
Phenmedipham	below LOQ	Captan	2.39 ± 53.4%
Tepraloxydim	below LOQ	Folpet	0.25 ± 7.9%
Prochloraz	below LOQ	Kresoxim-methyl	ND
Pyraclostrobin	0.0585 ± 80.4%	Fluazifop-P-butyl	ND
Prothioconazole	-	Chloridazon	ND
Fluazinam	ND		
Cycloxidum	-		

Using this method, one of the LC-amenable compounds (pyraclostrobin) could be detected in the bulb material. MRLs for pyraclostrobin in food range from 0.02-1 mg/kg in the EU, depending on the commodity (EU Pesticide database, http://ec.europa.eu/sanco_pesticides). In terms of safety, the average level detected in this study is within this range. Captan and folpet have MRLs from 0.02-5 mg/kg and 0.02-10 mg/kg, respectively. For folpet a level of up to 150 mg/kg is allowed for dried hops. Even though the average levels of these two compounds may be overestimated due to matrix problems, they are still within the MRL range for food. At present there are no MRLs specified for *Narcissus* bulbs as raw medicinal plant material. The level of pesticide residues allowed in bulbs will depend on how they will be processed after harvest, and how the galanthamine will be extracted. This will depend on the extraction method used by the extraction or pharmaceutical company responsible.

Overall the pesticides typically used in *Narcissus* cultivation do not seem to persist in or on the bulb at high concentrations. For quality control of *Narcissus* bulbs for contamination above MRLs values of pesticides commonly used in their cultivation the methods described here are satisfactory. However, for more sensitive analysis of pesticide residues, more sensitive equipment is needed. The extraction method should be optimized for the volatile GC-amenable compounds. From this study can be concluded that pesticide residues are not present at levels high enough to detect using ^{1}HNMR metabolomics.

Mycotoxins in *Narcissus pseudonarcissus* bulbs

Introduction

If a plant part is to be used as raw material for the manufacture of a pharmaceutical product, it is usually required to be free of any toxic/potentially harmful components such as pesticide residues, microbial contamination and mycotoxins. When a single chemical is to be extracted from the raw material, this may be less of a factor as when a crude preparation (e.g. powder, oil) is made out of the plant material. Nonetheless, in manufacturing pharmaceuticals, very strict quality control measures are in place and each impurity present in the final formulated product has to be analyzed, identified and known.

Mycotoxins are secondary metabolites produced by certain fungi. Their presence in food or medicinal plant material is problematic since they have been shown to be carcinogenic, teratogenic, tremorogenic, heamorrhagic and dermatitic to many organisms, and are known to cause hepatic carcinoma in humans (Aziz et al., 1998). Collectively, diseases caused by exposure to toxic fungal metabolites are collectively called mycotoxicoses (Bennett and Klich, 2003).

Mycotoxins occur in agricultural products all around the world. They can enter into the chain during the field stage, during storage or at a later stage. Mycotoxin contamination is especially problematic in conditions where shipping, handling or storage practices encourages mold growth (Bennett and Klich, 2003). For human food, three genera of fungi are mainly responsible for mycotoxin production, namely *Fusarium*, *Aspergillis* and *Penicillium* (Sweeney and Dobson, 1998). *Fusarium* mainly affects plants while growing in the field as a plant pathogen, and produce mycotoxins just before or after harvest, while *Aspergillis* and *Penicillium* are more common as contaminants in stages after harvest, such as during drying or storage.

All mycotoxins are low molecular weight natural products produced as secondary metabolites by filamentous fungi. Due to their large diversity of chemical structures and

biosynthetic origins mycotoxins can be hard to define and classify. Some of the most important mycotoxins are the aflatoxins (*Aspergillis* spp.), citrinin (*Penicillium*, *Aspergillus* and some other spp.), ergot alkaloids (*Claviceps*), fumonisins (*Fusarium*), Ochratoxin (*Aspergillis*), Patulin (*Penicillium* ssp), trichothecenes (group of more than 60 sesquiterpenoids, from various fungal families including *Fusarium*, *Myrothecium*, *Phomopsis*, *Stachybotrys*, *Trichoderma*, *Trichothecium* and others) and Zearalenone (*Fusarium* spp. on grains).

Biosynthesis of mycotoxins is determined by a wide array of physical, biological and chemical factors (D'Mello et al., 1998). Primary among these are time, temperature, humidity and physical damage by other organisms such as insects. These and other factors interact in complex ways to induce mycotoxin biosynthesis. Pesticide application has also been reported to affect mycotoxin production (D'Mello et al., 1998). Production of some *Fusarium* toxins may be increased by sub-lethal doses of certain fungicides and herbicides (Kabak et al., 2006).

As part of the quality control of *Narcissus* bulbs as raw material for the extraction of pharmaceutical product, Galanthamine, it is necessary to know whether the bulbs contain mycotoxins, and if so the levels at which they are present. *Narcissus* has long been cultivated as an ornamental flower bulb crop. In the Netherlands, various fungal pathogens attack *Narcissus* plants, and these are well studied for their effect on the appearance of flowers and yield of bulbs. Since *Narcissus* has not traditionally been cultivated for consumption or pharmaceutical use, the presence of mycotoxins has up to now not been of much importance. Some of the fungi that infect *Narcissus* bulbs in the Netherlands belong to genera that are known to produce mycotoxins. For example, *Fusarium oxysporium*, one of the main fungal pathogens in *Narcissus*, has been reported to produce mycotoxins (D'Mello et al., 1998). Members of the genus *Penicillium* also infect Narcissus and are also known to produce mycotoxins (Sweeney and Dobson, 1998).

The chemical diversity of mycotoxins poses a challenge for their detection and quantitation in plant material. Many methods have been developed for different classes of mycotoxins in various substrates. Mycotoxins are usually present at levels of around µg/kg, so detection methods need to be suitably sensitive (Shephard, 2008). Traditionally methods usually involve solvent extraction and cleanup followed by various chromatographic separation methods and detection by UV or mass spectrometry (Lin et al., 1998). Sensitive ELISA methods using antibodies have been developed for detection of certain mycotoxins in food (Trucksess and Scott, 2008). Another approach is to use PCR to detect mycotoxin-producing fungi in plant samples, targeting genes involved in mycotoxin biosynthetic pathways (Waalwijk et al., 2008).

With the strict control measures in place in the formulation of pharmaceutical products, the presence of mycotoxins is now an issue that needs to be investigated. Due to time constraints and lack of suitably sensitive equipment, *Narcissus* bulbs samples heavily infected with *Fusarium oxysporum* were sent to an analytical laboratory that routinely screens plant samples for the presence of mycotoxins. As a control a healthy *Narcissus* bulbs sample was also sent. The aim was to determine whether any mycotoxins were present in the infected tissue.

Methods

Two *Narcissus pseudonarcissus* cv. Carlton bulb samples (three healthy bulbs and three bulbs heavily infected with *Fusarium oxysporum*) were ground in liquid nitrogen. The finely ground samples were analyzed at the RIKILT Institute of Food Safety in Wageningen according to their standard LC-MS/MS based mycotoxin screening methods (Kokkonen and Jestoi, 2009).

Results

The results of the mycotoxin screening are shown in Table 9. The plant material was tested for the presence of thirty important mycotoxins. None of the mycotoxins were detected in the healthy bulb material above the limit of detection. In the *Fusarium*-infected bulbs one mycotoxin, Beauvericin, was detected at 0.195 mg/kg. Beauvericin was first isolated from *Baeuverina bassiana*, an insect-pathogenic fungus(Hamill et al., 1969). It is a cyclic hexadepsipeptide, consisting of alternating N-methyl-phenylalanine and D-α-hydroxy-isovaleryl-(2-hydroxy-3-methylbutanoic acid) units (Figure 4).

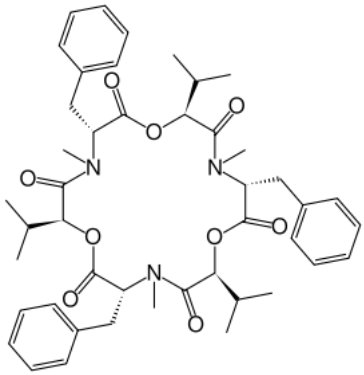

Figure 4. The chemical structure of Beauvericin.

Beauvericin can form stable complexes with cations (e.g. Sodium, potassium, calcium etc.) as well as some neutral or charged small molecules. These complexes are lipophilic and can be transported into a lipophilic phase (Hilgenfeld and Saenger, 1982). Beauvericin can also form dimeric complexes which can form channel pores in cell membranes through which ions can be transported (Hilgenfeld and Saenger, 1982; Kouri et al., 2003). Beauvericin was found to be toxic to brine shrimp when first isolated (Hamill et al., 1969). Antibacterial activity has been reported against various species (Castlebury et al., 1999). The compounds has been found active in various insecticidal bioassays, including mosquito larvae, blowfly and Colorado potato beetle (Grove and Pople, 1980; Gupta et al., 1991) . Toxicity to various vertebrate and invertebrate cell lines has also been reported (Çelik et al., 2010), with this bioactivity believed to be mainly due to the pore-forming properties of the beauvericin complexes (Tedjiotsop Feudjio et al., 2010). Beauvericin's activity against human cancer cell lines has aroused some interest in investigation as its use as a therapeutic lead compound (Tedjiotsop Feudjio et al., 2010).

A simple investigation to determine whether *Narcissus* bulbs infected with *Fusarium oxysporum* contains mycotoxins was carried out. The only mycotoxin found in this screening was the cyclic peptide beauvericin. This compound is toxic to human cells, and therefore its presence in raw pharmaceutical material should be known. Based on these results it is also recommended that only healthy bulbs are used for the extraction of galanthamine.

Table 9. Results of the mycotoxin screening. Compounds not detected (ND) are presented as less than the limit of detection (in mg/kg); a: <0.0005, b: <0.001, c: <0.005, d: <0.01, e: <0.02, f: <0.025, g: <0.04, h: <0.05, i: <0.1, j: <0.4.

Mycotoxin	**Healthy bulb (mg/kg)**	**Infected bulb (mg/kg)**
3&15-AcetylDON (sum of)	ND[h]	ND[h]
Aflatoxin B1	ND[a]	ND[a]
Aflatoxin B2	ND[a]	ND[a]
Aflatoxin G1	ND[a]	ND[a]
Aflatoxin G2	ND[b]	ND[b]
Agroclavine	ND[b]	ND[b]
Alternariol	ND[e]	ND[e]
Alternariol-methylether	ND[c]	ND[c]
Beauvericin	ND[c]	**0.195**
Citreoviridin	ND[e]	ND[e]
Citrinin	ND[d]	ND[d]
Deoxynivalenol	ND[i]	ND[i]
Diacetoxyscirpenol	ND[d]	ND[d]
Fumagillin	ND[g]	ND[g]
Fumonisin B1	ND[d]	ND[d]
Fumonisin B2	ND[d]	ND[d]
Fumonisin B3	ND[d]	ND[d]
HT2 toxin	ND[d]	ND[d]
Moniliformin	ND[h]	ND[h]
Mycophenolic acid	ND[h]	ND[h]
Neosolaniol	ND[e]	ND[e]
Nitropropionic acid	ND[d]	ND[d]
Ochratoxin A	ND[c]	ND[c]
Penicillic acid	ND[h]	ND[h]
Roquefortine C	ND[c]	ND[c]
Sterigmatocystin	ND[b]	ND[b]
T-2 Toxin	ND[d]	ND[d]
Verruculogen	ND[j]	ND[j]
ZON	ND[c]	ND[c]
α-Zearalenol	ND[f]	ND[f]
ß-Zearalenol	ND[h]	ND[h]

Chapter 8. Seasonal accumulation of major alkaloids in organs of *Narcissus pseudonarcissus* cv. Carlton

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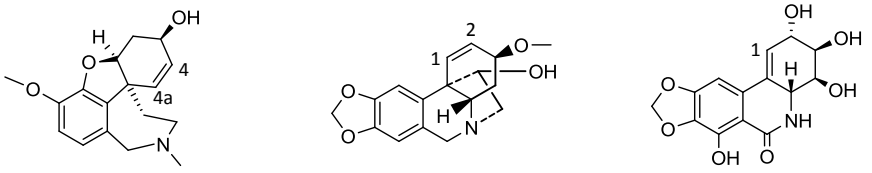
Abstract

Narcissus pseudonarcissus (L.) cv. Carlton is being cultivated for the extraction of galanthamine from the bulbs. After galanthamine, haemanthamine and narciclasine are the next most abundant alkaloids in this cultivar. Both these compounds are promising chemical scaffolds for potential drugs. For further research and drug development, a reliable supply of these compounds will be needed. In this study a field experiment was conducted to investigate the levels of galanthamine, haemanthamine and narciclasine in plants of *N. pseudonarcissus* cv. Carlton. In a field experiment alkaloids in the bulbs, leaves and roots were analyzed by quantitative ^{1}H NMR to monitor the variations during the growing season. Major primary and secondary metabolites were identified in the various plant parts. Multivariate data analysis was performed on the ^{1}H NMR spectra to see how metabolites changed in the plant organs over time. The results show that the leaves have relatively high concentrations of the alkaloids before flowering. The bulbs had lower concentrations of the compounds of interest but would have a higher total yield of alkaloids due to bigger biomass. Since *Narcissus pseudonarcissus* is already being cultivated for the extraction of galanthamine, it represents a good source of the other compounds.

Introduction

Daffodils (*Narcissus* species) are one of the major ornamental flower crops in the Netherlands, with large volumes of bulbs being planted every year. Since recently one of the varieties, *Narcissus pseudonarcissus* (L.) cv. Carlton, is being cultivated for the extraction of the compound galanthamine from the bulbs (Berkov et al., 2009). This cultivar was chosen because of the relatively high concentration of galanthamine in the bulbs, the large bulb size and the availability of large volumes of planting stocks of the bulbs (Kreh, 2002). Galanthamine is an alkaloid that occurs in several species of the Amaryllidaceae plant family. Due to its effects on the human brain (see Chapter 3) it is now a registered drug for the symptomatic treatment of early stage Alzheimer's disease (Sramek et al., 2000; Heinrich and Teoh, 2004). More than 300 alkaloids have been isolated from the genus of *Narcissus*, and most of them possess some biological activities (Bastida et al., 2006). Galanthamine is usually reported as the major alkaloid in the bulbs of *N. pseudonarcissus*, followed by haemanthamine as the second-most abundant (Figure 1), and a number of other minor alkaloids such as homolycorine, lycoramine and *O*-methyllycorenine (Kreh et al., 1995; Gotti et al., 2006). More than 20 alkaloids have been isolated from the *Narcissus* cultivar "Carlton" (Bastida et al., 2006).



galanthamine haemanthamine narciclasine

Figure 1. Structures of galanthamine, haemanthamine and narciclasine. The protons used for quantitative NMR analysis are indicated on the structures.

Narciclasine is an isocarbostyril compound similar in structure to the alkaloid lycorine. Although not a basic compound, it is usually still included in the group of Amaryllidaceae alkaloids (Bastida et al., 2006). Narciclasine was first identified in *N. pseudonarcissus* by Piozzi and Marino (Piozzi et al., 1969). Although narciclasine is known to occur in *N. pseudonarcissus* cv. Carlton, this compound is usually not included in studies on the alkaloid profile of this cultivar. This is due to the extraction conditions in these analyses (usually involving acid-base extraction of alkaloids) being selective for basic compounds. Consequently, studies that report changes in the alkaloid levels in *Narcissus* during the course of the growing season do not include narciclasine (Kreh, 2002).

Haemanthamine also has interesting properties, including inhibition of protein synthesis, antiretroviral, antiparasite and antimalarial activity, as well as cytotoxicity against various cancer cells (Bastida and Viladomat, 2002; Sener et al., 2003; Szlavik, 2004; Osorio et al., 2010). Haemanthamine's ability to induce apoptosis in cancer cells has attracted much attention, and the compound has potential as a chemical scaffold for producing derivatives that could become future cancer drugs (McNulty et al., 2007; Evidente and Kornienko, 2009; Van Goietsenoven et al., 2010). Narciclasine also has the ability to induce apoptosis in various human cancer cell lines (Dumont et al., 2007). This compound and some of its semi-synthetic derivatives are also promising candidates for cancer drugs, particularly against apoptosis-resistant cancer cells (Ingrassia et al., 2009). For further research, clinical trials and beyond a stable supply of these compounds would be needed. This is often a limitation in drug development with natural products (McChesney et al., 2007). Since *N. pseudonarcissus* cv. Carlton is already being cultivated for the extraction of galanthamine, it represents an available and well-established source of the other compounds as well.

In this study a field experiment was conducted to investigate the levels of galanthamine, haemanthamine and narciclasine in plants of *N. pseudonarcissus* cv. Carlton. Plants were harvested at different time points throughout the growing season. The bulbs, leaves and roots were analyzed by quantitative ^{1}H NMR to monitor the variations in the major alkaloids through time. Various primary and secondary metabolites were identified in different parts of the plant. Multivariate data analysis was performed on the ^{1}H NMR spectra to see how detectable metabolites changed in the plant organs over time.

Materials and methods

Chemicals and solvents

For the NMR analysis methanol-d_4 (99.80%) from Cambridge Isotope Laboratories (Andover, MA, USA), and phosphate (KH_2PO_4) buffer (pH 6.0) in deuterium oxide (CortecNet, Voisins-Le-Bretonneux, France) containing 0.01% trimethylsilylproprionic acid sodium salt-d_4 (TMSP, w/w) as an internal standard for quantitation and calibration of chemical shift was used.

Plant material

Bulbs of *Narcissus pseudonarcissus* L. (Amaryllidaceae) cv. Carlton were planted in November 2009 in sandy soil in Lisse, the Netherlands. Three plots were planted, each consisting of two rows of 11, and two rows of 10 bulbs (total 42 bulbs). The rows were planted 18 cm apart, and each plot was surrounded by an edge of open space of 70 cm.

All plots received the standard amount of nitrogen and potassium fertilizers, consisting of 110 kg/hectare of Kalksalpeter ($Ca(NO_3)_2$ with 19% Ca and 15.5% N, consisting of 14.4% N-NO_3, 1.1% N-NH_4) and 150 kg/hectare Patentkali (K_2SO_4 and $MgSO_4$, 30% K as K_2O, 10% Mg as MgO and 42% S as SO_3).The bulbs in this experiment did not receive the hot water treatment before planting.

Plants were harvested throughout the growing season. Twenty-four plants were harvested at five different time-points: when shoots had emerged and were about 10cm above ground (A), before flowering (B), during full flowering (C), after flowering (D) and at normal harvest time after shoot senescence (D). Bulbs, roots and leaves were processed for extraction on the same day of harvest. A summary of the harvest times and dates are given below in Table 1.

Table 1. Summary of the time when the *Narcissus pseudonarcissus* cv. Carlton plants were harvested in the field.

Time point	Description	Date lifted
A	Shoots emerge 10 cm above ground	16/03/2010
B	Before flowering	06/04/2010
C	Full flowering	03/05/2010
D	After flowering	21/05/2010
E	Shoots senescing, end of season (before lifting)	08/07/2010

Sample preparation and ^{1}H NMR measurement

The plants were rinsed to remove soil particles. The roots, bulbs and leaves were separated by cutting with a sharp blade. The basal plates were removed from the bulbs to aid grinding. The different organ samples from each plant were frozen in liquid nitrogen and individually ground to fine powder in a Waring laboratory blender (Waring Products Inc., Torrington, CT, USA). The ground plant material was freeze-dried and kept at -80°C until analysis. Extraction of *N. pseudonarcissus* cv. Carlton bulbs, roots and leaves and ^{1}H NMR measurements were carried out as described in Lubbe et al. (2010). For the bulbs, twenty-four biological replicates were extracted, and for the leaves and roots there were twelve biological replicates at each time point. Fifty milligram of freeze-dried plant material was weighed into 2 mL microtubes and extracted with 1.5 mL of a mixture of phosphate buffer (pH 6.0) in deuterium oxide containing 0.01% trimethylsilylproprionic acid sodium salt-d_4 (TMSP, w/w) and methanol-d_4 (1:1). Samples were ultrasonicated for 30 minutes, followed by centrifugation at 13000 rpm for 10 minutes. An aliquot of 1 mL of the supernatant was collected and 800 µL transferred to 5 mm NMR tubes for ^{1}H NMR measurement. ^{1}H

NMR spectra were recorded with a Bruker AV 600 spectrometer (Bruker, Karlsruhe, Germany). For each sample 64 scans were recorded using the following parameters: 0.167 Hz/point, pulse width (PW) 4.0 μs and relaxation delay (RD) = 5.0 s. FIDs were Fourier transformed with LB = 0.3 Hz. Manual phase adjustment and baseline correction were applied as well as calibration with internal standard TMSP to 0.0 ppm.

Data processing and multivariate data analysis

For quantitative NMR analysis of galanthamine, integration of the doublet proton signal at δ 6.17 (galanthamine H-4a) was performed. The ratio of this integral to that of the internal standard was used to calculate the amount of galanthamine per milligram material. Quantitative analysis of haemanthamine and narciclasine was performed in the same way by integration of the doublet proton signal at δ 6.48 (haemanthamine H-1) and multiplet proton signal at δ 6.22 (narciclasine H-1), respectively.

For multivariate data analysis, ^{1}H NMR spectra were automatically binned by AMIX software (v.3.7, Biospin, Bruker). Spectral intensities were scaled to total intensity and the region of δ 0.32-10.0 was reduced to integrated regions ("buckets" or " bins") of 0.04 ppm each. The regions δ 4.7-5.0 and δ 3.30-3.34 were excluded from the analysis because of the presence of the residual water and methanol signal, respectively. Principal component analysis (PCA) was performed with SIMCA-P software (v. 12.0 Umetrics, Umeå, Sweden) using the Pareto scaling method.

Results

Quantitation of major alkaloids in bulbs, stems and roots

The galanthamine concentration in the bulbs was determined using a quantitative ^{1}H NMR method developed previously (Lubbe et al., 2010). The area under the doublet signal at δ 6.17 belonging to H-4a of galanthamine was used for quantitation. The doublet signal at δ 6.52 (H-1) of haemanthamine and the multiplet signal at δ 6.22 (H-1) of narciclasine were used in a similar manner for quantitation of these compounds. In some leaf samples the H-1 doublet of haemanthamine was overlapped with other signals, in which case the broad singlet at δ 5.99 (OCH_2O) was used for quantitation. The same signals, where present, were used for quantitation of these metabolites in the leaf and root samples.

Figure 2 shows the results of the quantitative analysis of galanthamine, haemanthamine and narciclasine in the bulbs, leaves and roots of the *Narcissus* plants. Galanthamine was the major alkaloid in all the bulb samples, followed by haemanthamine and narciclasine. The average bulb galanthamine concentration increased from the first time

point to a maximum before flowering. The concentration decreased over the next two time points but showed a slight increase again at senescence of the aerial parts. Haemanthamine, the second most abundant alkaloid showed a similar pattern, with the highest average concentration just before flowering. Narciclasine in the bulbs was highest on average before flowering and during flowering, and thereafter steadily decreased until the end of the season. In the leaves haemanthamine was the major alkaloid in the first three time points. The concentration varied through the season with a maximum before flowering. Galanthamine and narciclasine were present at roughly the same levels in the leaves, with steady levels until full flowering followed by a decrease after flowering. These three compounds could not be detected in the senescent leaves. Galanthamine and haemanthamine were present in the root, with galanthamine at higher concentrations in the first two time points and haemanthamine significantly higher in the final time point. Narciclasine, if present in the roots, was at levels too low to detect.

Metabolite identification

Identification of metabolites was done with the aid of two-dimensional NMR experiments (COSY, J-Resolved and HMBC), as well as comparison of signals with an in-house metabolite database and previously reported data (Verpoorte et al., 2007; Kim et al., 2010). Bulb metabolites were similar to what has previously been reported in studies using this NMR extraction method (Lubbe et al., 2010, 2011), and a GC-MS based analysis of *N. pseudonarcissus* cv. Carlton bulb metabolites (Berkov et al., 2011). Signal assignments are summarized in Table 2. The bulb spectra were mostly similar in terms of the metabolites present, but the levels of some signals fluctuated between time points.

In the leaf samples differences in metabolites were clearly seen between time points, with more qualitative differences between the different stages (Figure 3). In all spectra intense signals of the carbohydrates sucrose, fructose and glucose were present. The alkaloid levels were highest at the earlier time-points and decreased over the course of the season. Major signals were seen in the leaf spectra at around δ 4.28, δ 2.70 and δ 2.43 and were assigned to malic acid. These signals are shifted in the later time points (Figure 3b), a phenomenon known to occur with malic acid signals due to changes in concentration and pH (Verpoorte et al., 2007). Other major primary metabolites were assigned with the aid of two-dimensional NMR experiments (COSY, J-Resolved and HMBC), as well as comparison of signals with an in-house metabolite database and previously reported data (Verpoorte et al., 2007; Kim et al., 2010). The amino acids alanine, threonine, valine, isoleucine, glutamine, aspartic acid, phenylalanine and tryptophan were identified in the leaf samples.

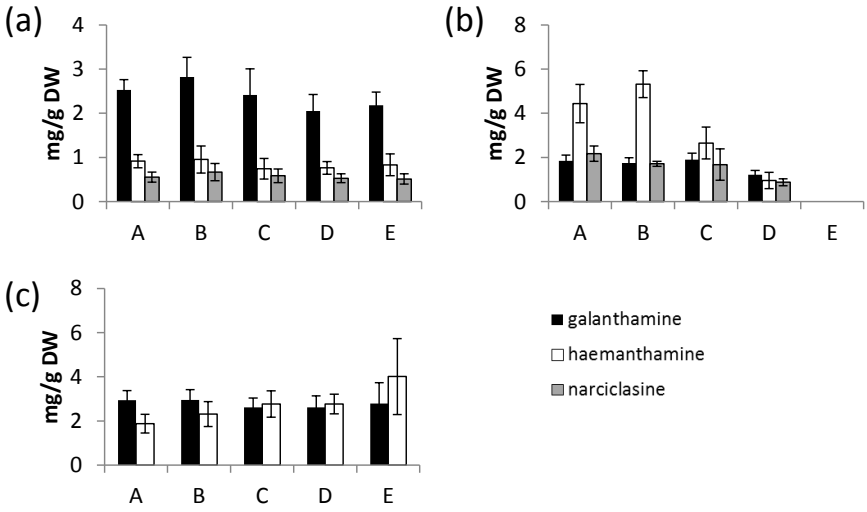


Figure 2. Results of quantitative ^{1}H NMR analysis of the major alkaloids in *Narcissus pseudonarcissus* cv. Carlton (a) bulbs, (b) leaves and (c) roots at different time-points during the growing season (mean mg/g ± SD, n=24 for bulbs and n=12 for leaves and roots). A: shoots emerge, B: before flowering, C: full flowering, D: after flowering, E: shoot senescence.

The aromatic region of the leaf spectra contained various signals not seen in the bulb spectra (Figure 4). One of these was identified as chlorogenic acid, a phenolic compound commonly found in photosynthetic tissue (Verpoorte et al., 2007). Signals similar to chlorogenic acid, but shifted slightly downfield, match those reported for 5-O-feruloylquinic acid, which has a methoxy group instead of a hydroxyl group at C-3' (Leiss et al., 2009). The flavonol glycoside rutin was identified by its characteristic doublet and double-doublet signals of the A ring at δ 6.51 and δ 6.31 and B ring at δ 7.72, 7.67, 6.99 of the flavonol moiety, as well as the doublet signal of the rhamnosyl moiety at δ1.10 with doublet (J = 6.4 HZ) (Zhi et al., 2012). Signals indicating the presence of two kaempferol analogues and two quercetin analogues were detected. Some signals were seen only in the first time-point, namely those assigned to the alkaloid precursor 4- hydroxyphenylpyruvate and an unidentified phenylpropanoid. A triplet signal at 1.19, assigned to 1-*O*- ethyl glucoside was seen only in leaf samples in the after flowering stage. Metabolites assigned in the leaf samples are summarized in Table 3.

Table 2. ^{1}H Chemical shifts (δ) and coupling constants (Hz) of *Narcissus pseudonarcissus* bulb metabolites in methanol-d_4-KH_2PO_4 in D_2O at pH 6.0.

No.	Metabolite	Chemical shift (δ) and coupling constant (Hz)
1	Fumaric acid	8.47 (s)
2	Phenylalanine	7.42-7.33 (m), 3.09 (dd) J=8.3, 14.8
3	Tyrosine	7.18 (d) J=8.4, 6.85 (d) J=8.4
4	4-hydroxyphenylpyruvate	7.13 (d) J=8.4, 6.73 (d) J=8.4, 3.02 (d) J=13.6, 2.98 (d) J=13.6
5	Lycorenine	7.06 (s), 7.04 (s), 6.02 (s), 5.73 (brs)
6	Cis-aconitic acid	7.03 (s)
7	Galanthamine	6.94 (d) J=8.4, 6.88 (d) J=8.4, 6.16 (d) J=10.5, 6.06 (dd) J=10.5, 5.0, 2.86 (s)
8	Haemanthamine	7.06 (s), 6.71 (s), 6.51 (d) J=10.3, 6.36 (dd) J=10.3, 5.0, 5.97 (brs), 6.22 (m), 6.09 (2d) J=4.5, 6.73 (s), 4.39 (m), 4.32 (m)
9	Narciclasine	6.22 (m), 6.09 (2d) J=4.5, 6.73 (s), 4.39 (m), 4.32 (m)
10	Raffinose	5.55 (d) J=3.8, 5.27 (d) J=3.8
11	Sucrose	5.41 (d) J=3.8, 4.17 (d) J=8.7, 4.03 (t) J=8.3, 3.78-3.83 (m), 3.75 (t) J=9.5, 3.66 (s), 3.51 (dd) J=9.9, 3.9, 3.43 (t) J=9.5
12	Arabinose	5.23 (d) J=3.7
13	Maltose	5.17 (d) J=3.8, 5.40 (d) J=3.9
14	Mannose	5.14 (d) J=1.5
15	Rhamnose	5.11 (d) J=1.5, 4.87 (d) J=0.7, 1.28 (t) J=6.5
16	Glucose	4.58 (d) J=7.9 (β-anomer), 5.19 (d) J=3.8 (α-anomer), 3.20 (dd) J=8.8, 8.9
17	Choline	3.21 (s)
18	Ethanolamine	3.12 (t) J=5.3
19	Asparagine	3.94 (dd) J=8.0, 4.0, 2.95 (dd) J=17.0, 3.8, 2.81 (dd) J=17.0, 8.2
20	Aspartic acid	2.82 (dd) J=17.0, 8.5, 2.63 (dd) J=17.0, 9.5
21	Citric acid	2.71 (d) J=15.8, 2.56 (d) J=15.8
22	Malic acid	2.68 (dd) J=15.7, 3.4, 2.36 (dd) J=15.7, 10.4, 4.28 (dd) J-10.4, 3.2
23	Glutamic acid	2.39 (td) J=7.1, 2.5, 2.10-2.18 (m)
24	Ornithine	3.24 (t) J=8.0, 1.92 (m), 1.65-1.78 (m), 3.71 (t) J=5.8
25	Acetic acid	1.91 (s)
26	Alanine	1.49 (d) J=7.2
27	Valine	1.06 (d) J=7.0, 1.01 (d) J=7.04
28	Isoleucine	1.03 (d) J=7.1, 0.96 (t) J=7.4
29	1-*O*-ethyl glucoside	1.19 (t) J=7.0
30	Threonine	1.34 (d) J=6.6, 4.22 (m)
31	Fatty acids	1.31 (brs), 2.18 (t) J=7.4, 1.56 (m), 0.89 (t) J=7.4, 5.40 (m)

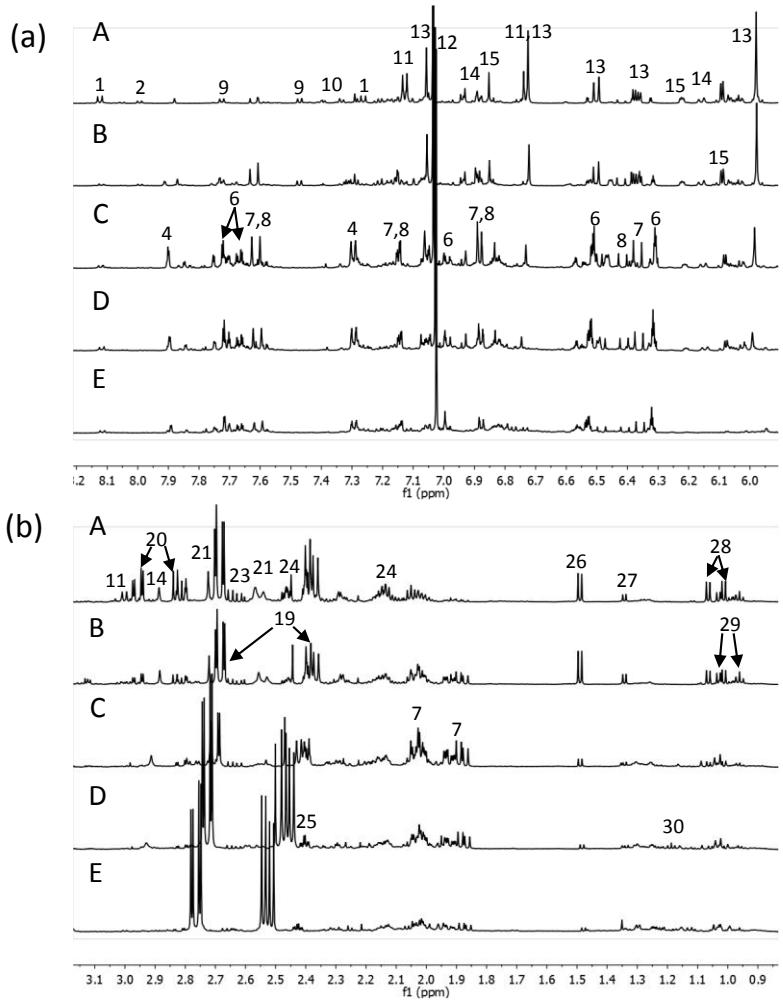


Figure 3. Stacked ^{1}H NMR spectra from (a) δ 6.0 – δ 8.2 and (b) δ 3.2 – δ 0.9 of representative *Narcissus pseudonarcissus* cv. Carlton leaf extracts from different time-points in the growing season. A: shoots emerge, B: before flowering, C: full flowering, D: after flowering, E: shoot senescence. Assigned signals are numbered as indicated in Table 3. 1: kaempferol analogue 1, 2: kaempferol analogue 2, 4: quercetin analogue 1, 6: rutin, 7: chlorogenic acid, 8: 5-*O*-feruloylquinic acid, 9: tryptophan, 10: phenylalanine, 11: 4-hydroxyphenylpyruvate, 12: cis-aconitic acid, 13: haemanthamine, 14: galanthamine, 15: narciclasine, 16: sucrose, 19: malic acid, 20: conjugated malic acid, 21: citric acid, 23: aspartic acid, 24: glutamine, 25: glutamic acid, 26: alanine, 27: threonine, 28: valine, 29: isoleucine, 30: 1-*O*-ethyl glucoside.

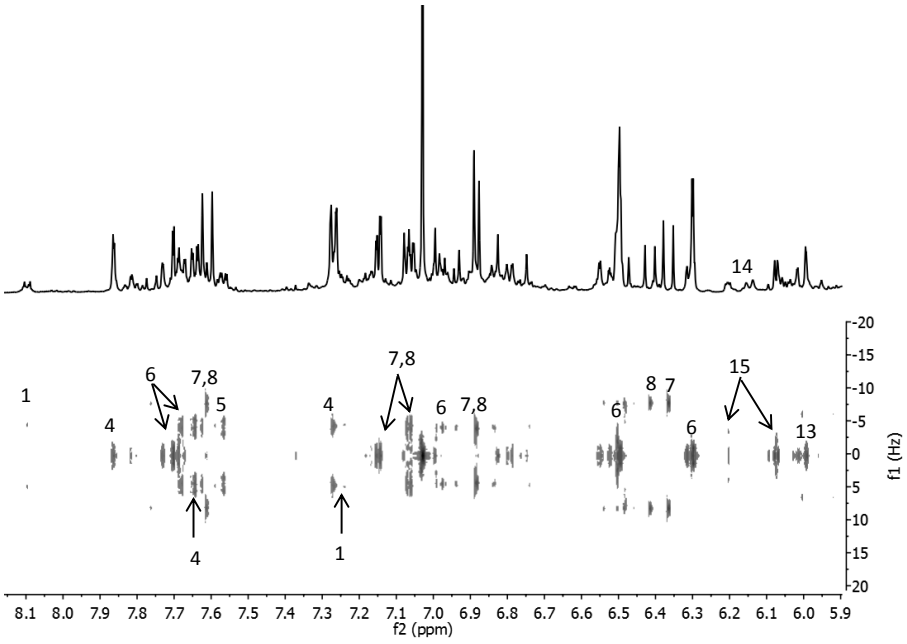


Figure 4. J-resolved spectrum of a representative *Narcissus pseudonarcissus* leaf extract in KH_2PO_4 buffer and Methanol-d_4 (1:1) pH 6.0, harvested around the time of flowering. Assigned signals are numbered as indicated in Table 3; 1: kaempferol analogue 1, 4: quercetin analogue 1, 5: quercetin analogue 2, 6: rutin, 7: chlorogenic acid, 8: 5-*O*-feruloylquinic acid, 13: haemanthamine, 14: galanthamine, 15: narciclasine.

The metabolite profiles of root samples were inspected and several primary and secondary metabolites were assigned (Table 4). Haemanthamine and galanthamine were present in all the samples, as well as cis-aconitic acid. A kaempferol analogue was seen in the aromatic part of the spectrum in all time-points, and primary metabolites malic acid, fructose, glucose and sucrose were present in all samples. Signals matching those of trigonelline (Lopez-Gresa et al., 2010) were seen in the first two time-points, together with the amino acids tryptophan and phenylalanine. The amino acids threonine, isoleucine and valine were also present in earlier samples. Signals tentatively assigned to a quercetin analogue occurred in leaf samples around the time of flowering. At leaf senescence, many signals not seen at any other time points were present. In the aromatic region of the spectra, characteristic phenolic signals were observed at relatively high

levels. Fatty acid signals were observed in the last three time-points, with the signals the most intense at the senescence time point.

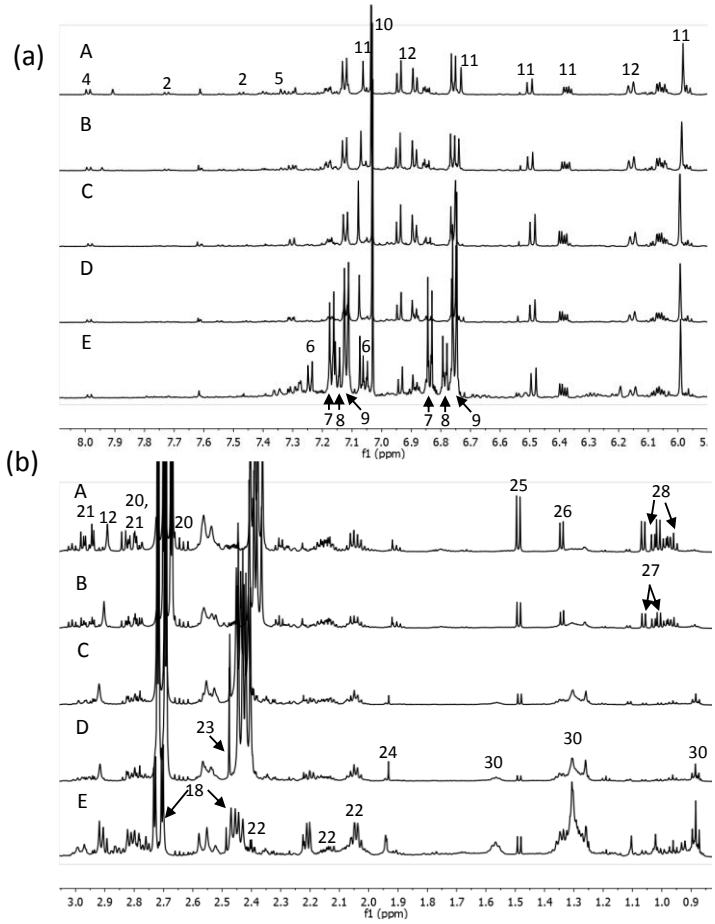


Figure 5. Stacked ^{1}H NMR spectra from (a) δ 8.1 – δ 5.9 and (b) δ 3.0 – δ 0.8 of representative *Narcissus pseudonarcissus* cv. Carlton root extracts from different time-points in the growing season, measured in KH_2PO_4 buffer and Methanol-d_4 (1:1) pH 6.0. A: shoots emerge, B: before flowering, C: full flowering, D: after flowering, E: shoot senescence. Assigned signals are numbered as indicated in Table 4; 2: tryptophan, 4: kaempferol analogue, 5: phenylalanine, 6: phenolic 1, 7: tyrosine, 8: phenolic 2, 9: phenolic 3, 10: *cis*-aconitic acid, 11: haemanthamine, 12: galanthamine, 18: malic acid, 20: aspartic acid, 21: asparagine, 22: glutamine, 23: succinic acid, 24: acetic acid, 25: alanine, 26: threonine, 27: valine, 28: isoleucine, 30: fatty acids.

Table 3. ^{1}H Chemical shifts (δ) and coupling constants (Hz) of *Narcissus pseudonarcissus* leaf metabolites in Methanol-d4-KH2PO4 in D2O at pH 6.0.

No.	Metabolite	Chemical shift (δ) and coupling constant (Hz)
1	Kaempferol derivative 1	8.09 (d) J=8.9, 7.24 (d) J=8.9, 6.45 (d) J=2.0, 6.29 (d) J=2.0, 5.17 (7.6)
2	Kaempferol derivative 2	8.02 (d) J=8.73, 6.98 (d) J=8.73
3	Phenylpropanoid	7.76 (d) J=16.0, 6.48 d J=16.0
4	Quercetin derivative 1	7.86 (d) J=2.0, 7.68 (dd) J=2.0, 9.9, 7.26 (d) J=9.9
5	Quercetin derivative 2	7.69 (d) J=8.8, 7.56 (dd) J=8.8, 2.0, 6.97 (d) J=8.8
6	Rutin	7.72 (d) J=2.1, 7.67 (dd) J=2.1, 8.5, 6.99 (d) J=8.5, 6.51 (d) J=2.0, 6.31 (d) J=2.0, 5.07 (d) J=7.0, 4.53 (d) J=2.6, 1.1 (d) J=6.4
7	Chlorogenic acid (5-*O*-caffeoylquinic acid)	7.61 (d) J=15.9, 6.37 (d) J=15.6, 7.14 (d) J=1.0, 7.06 (dd) J=1.0, 8.9, 6.88 (d) J=8.9, 5.32 (t of d), 4.10 (d), 3.98, 2.02, 2.03, 1.93 (d of t), 1.88 (dd)
8	5-*O*-feruloylquinic acid	7.61 (d) J=15.9, 6.41 (d) J=15.9, 7.15 (d) J=1.8, 7.05 (dd) J=8.0, 1.8, 6.88 (d) J=8.0
9	Tryptophan	7.72 (d) J=7.9, 7.47 (d) J=8.13, 7.29 (s), 7.21 (t) J=7.5
10	Phenylalanine	7.40 (m), 7.34 (m)
11	4-Hydroxyphenylpyruvate	7.13 (d) J=8.4, 6.73 (d) J=8.4, 3.02 (d) J-13.2, 2.98 (d) J=13.2
12	*cis*-Aconitic acid	7.03 (s)
13	Haemanthamine	7.08 (s), 6.75 (s), 6.49 (d) J=10.0, 6.40 (dd) J=10.0, 5.0, 5.99 (brs)
14	Galanthamine	6.94 (d) J=8.2, 6.89 (d) J=8.2, 6.16 (d) J=10.0, 6.06 (dd) J=10.0, 5.0, 2.88 (s)
15	Narciclasine	6.80 (s), 6.20 (m), 6.07 (2d) J=4.5
16	Sucrose	5.41 (d) J=3.7, 3.80 (m), 3.51 (dd) J=10.0, 3.76, 3.74 (t) J=9.5, 3.44 (t) J=9.5
17	Glucose	5.19 (d) J=3.8, 4.58 (d) J=7.8, 3.20 (dd) J=7.8, 7.4
18	Fructose	4.07 (m), 4.02 (dd) J=13.8, 1.0, 3.94 (m), 3.85 (dd) J=9.8, 3.6, 3.79 (m), 3.70 (d) J=11.8, 3.52 (d) J=11.8
19	Malic acid	4.28 (dd) J=9.1, 3.5, 2.71 (dd) J=15.6, 3.5, 2.43 (dd) J=15.6, 9.1
20	Conjugated malic acid	4.25 (dd) J=8.0, 4.0, 2.96 (dd) J=16.0, 4.0, 2.82 (dd) J=16.0, 8.0
21	Citric acid	2.71 (d) J=15.8, 2.56 (d) J=15.8
22	Ethanolamine	3.12 (t) J=5.20
23	Aspartic acid	2.82 (dd) J=17.0, 8.5, 2.64 (dd) J=17.0, 10.0
24	Glutamine	2.46 (t of d), 2.16-2.10 (m)
25	Glutamic acid	2.39 (t of d) J=7.1, 2.10-2.18 (m)
26	Alanine	1.48 (d) J=7.21
27	Threonine	1.34 (d) J=6.6
28	Valine	1.01 (d) J=6.8, 1.06 (d) J=6.8
29	Isoleucine	1.03 (d) J=7.1, 0.96 (t) J=7.4
30	1-*O*-ethyl glucoside	1.19 (t) J=7.09

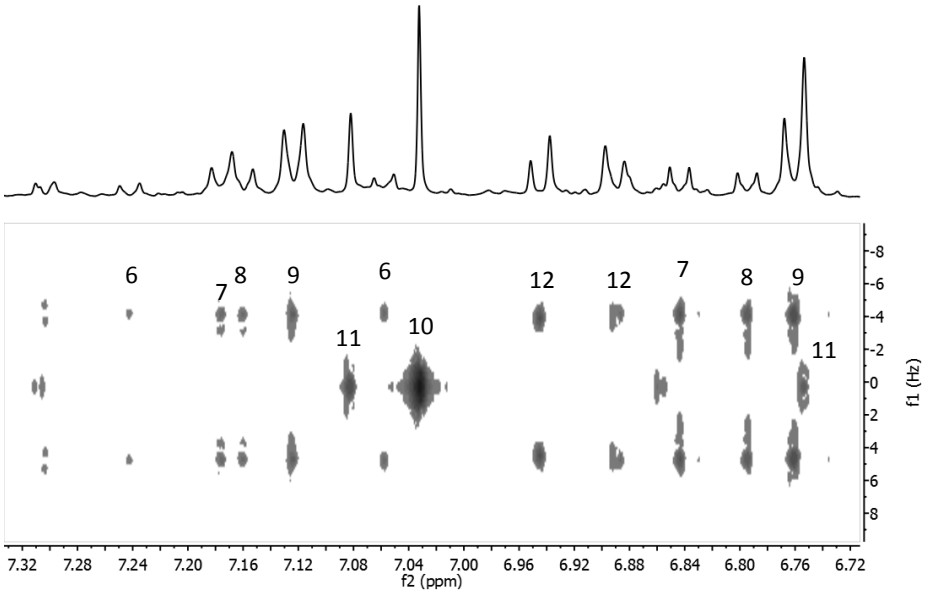


Figure 6. J-resolved spectrum of a representative *Narcissus pseudonarcissus* root extract in KH_2PO_4 buffer and Methanol-d_4 (1:1) pH 6.0, harvested at time of leaf senescence. Assigned signals are numbered as indicated in Table 4; 6: phenolic 1, 7: tyrosine, 8: phenolic 2, 9: phenolic 3, 10: *cis*-aconitic acid, 11: haemanthamine, 12: galanthamine.

Multivariate data analysis

The recorded ^{1}H NMR spectra of the bulbs, stems and roots of *N. pseudonarcissus* harvested at different time-points were submitted to Principal Component Analysis (PCA) to obtain an overview of the variation in metabolite profiles between time points. For the bulbs the first two PCs of the model together accounted for 79.2% of the variance in the dataset. The first four time-points were separated along PC1, while PC2 separated the last time point from the others (Figure 7a). There were some overlaps between the first four time-points, although most of A and B samples occurred on the negative side of PC1, and most of C and D samples on the positive side of PC1. The variance in the leaf ^{1}H NMR spectra was well described by the PCA model of these samples. The first two PCs accounted for 84.1% of the variance, and clear separation and grouping of the time-points could be seen in the resulting score plot shown in fig. 7b. PC1 separated time point A and B from the rest of the groups, and C, D and E were separated along PC2.

Table 4. ^{1}H Chemical shifts (δ) and coupling constants (Hz) of *Narcissus pseudonarcissus* root metabolites in Methanol-d_4-KH_2PO_4 in D_2O at pH 6.0.

No.	Metabolite	Chemical shift (δ) and coupling constant (Hz)
1	Trigonelline	9.14 (s), 8.87 (m), 8.10 (dd) J=7.5, 6.5, 4.42 (s)
2	Tryptophan	7.72 (d) J=7.9, 7.47 (d) J=8.13, 7.29 (s), 7.21 (t) J=7.5
3	Quercetin analogue	7.60 (d) J=1.84, 7.54 (dd) J=8.55, 1.85
4	Kaempferol analogue	7.99 (d) J=8.2, 5.96 (d) J=8.2
5	Phenylalanine	7.40 (m), 7.34 (m)
6	Unidentified Phenolic 1	7.24 (d) J=8.8, 7.06 (d) J=8.8
7	Tyrosine	7.18 (d) J=8.6, 6.84 (d) J=8.6
8	Unidentified Phenolic 2	7.16 (d) J=8.6, 6.80 (d) J=8.6
9	Unidentified Phenolic 3	7.12 (d) J=8.5, 6.76 (d) J=8.5
10	Cis-aconitic acid	7.04 (s)
11	Haemanthamine	7.08 (s), 6.75 (s), 6.49 (d) J=10.0, 6.40 (dd) J=10.0, 5.0, 5.99 (brs)
12	Galanthamine	6.94 (d) J=8.2, 6.89 (d) J=8.2, 6.16 (d) J=10.0, 6.06 (dd) J=10.0, 5.0, 2.88 (s)
13	Sucrose	5.41 (d) J=3.7, 3.80 (m), 3.51 (dd) J=10.0, 3.76, 3.74 (t) J=9.5, 3.44 (t) J=9.5
14	Glucose	5.19 (d) J=3.8, 4.58 (d) J=7.8, 3.20 (dd) J=7.8, 7.4
15	Fructose	4.07 (m), 4.02 (dd) J=13.8, 1.0, 3.94 (m), 3.85 (dd) J=9.8, 3.6, 3.79 (m), 3.70 (d) J=11.8, 3.52 (d) J=11.8
16	Arabonise	5.22 (d) J=3.75
17	Mannose	5.14 (d) J=1.5
18	Malic acid	4.28 (dd) J=9.1, 3.5, 2.71 (dd) J=15.6, 3.5, 2.43 (dd) J=15.6, 9.1
19	Ethanolamine	3.12 (t) J=5.20
20	Aspartic acid	2.82 (dd) J=17.0, 8.5, 2.64 (dd) J=17.0, 10.0
21	Asparagine	3.94 (dd) J=8.0, 4.0, 2.95 (dd) J=16.0, 4.0, 2.82 (dd) J=16.7, 8.0
22	Glutamine	2.46 (t of d), 2.16-2.10 (m)
23	Succinic acid	2.47 (s)
24	Acetic acid	1.92 (s)
25	Alanine	1.48 (d) J=7.21
26	Threonine	1.34 (d) J=6.6
27	Valine	1.01 (d) J=6.8, 1.06 (d) J=6.8
28	Isoleucine	1.03 (d) J=7.1, 0.96 (t) J=7.4
29	1-*O*-ethyl glucoside	1.19 (t) J=7.09
30	Fatty acids	1.30 (brs), 0.88 (t) J=7.2, 1.56 (m), 2.20 (t) J=7.6

The results of the PCA of the roots samples are shown in Figure 7c. The first two principal components accounted for 68.7% of the variance for the original dataset. PC1 separated the senescence time-point from the other time points, which were separated along PC2. The first time-point (A) was well separated from the others, but as in the bulb samples there was some overlap between the groups of time-point B, C and D along PC2.

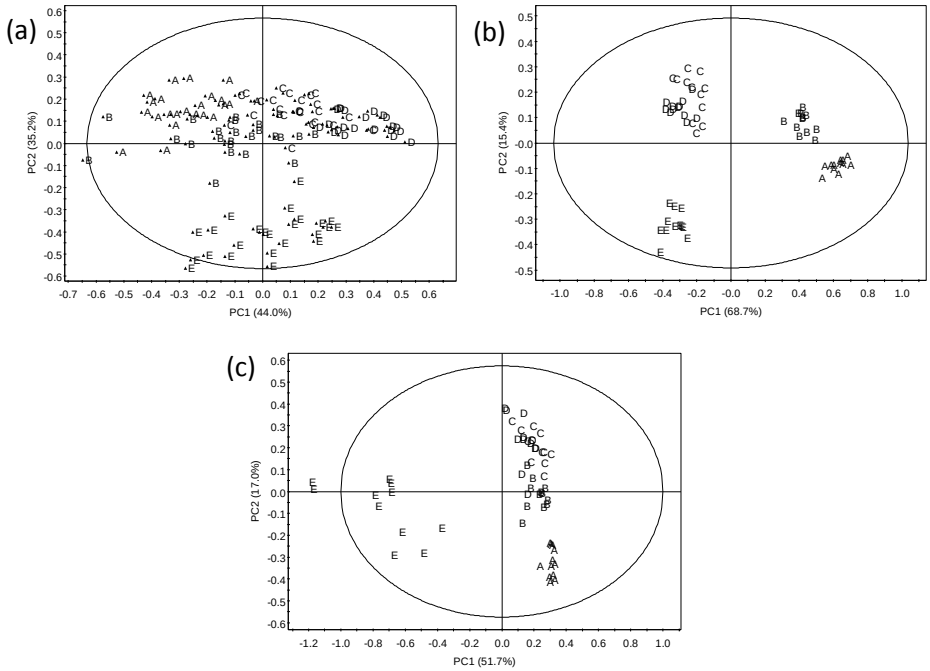


Figure 7. Score scatter plots of Principal Component Analysis (PC1 versus PC2) of *Narcissus pseudonarcissus* bulbs (a), leaf (b) and root (c) samples harvested at different time points throughout the growing season. A: shoots emerge, B: before flowering, C: full flowering, D: after flowering, E: shoot senescence.

The difference between the metabolite profiles of the different organs of *N. pseudonarcissus* was further investigated to see which metabolites were responsible for the groupings observed in the PCA results. The NMR samples of each organ were grouped according to time-points. A supervised data analysis method, Partial Least Squares Discriminant Analysis (PLS-DA) was used to correlate NMR signals to the different time-point groups. The results of the PLS-DA analysis are shown in Figure 8.

Bulb ^{1}H NMR signals (the X variables in this analysis) correlated to the different time-points (Y variables) could be read off the loading scatter plot (Figure 8b). With the aid of a VIP plot the signals most important for the discrimination of the groups in the model could be identified. For the bulbs, signals important in the first two time-points belonged to phenylalanine, alanine, valine, and ornithine. Signals belonging to unsaturated fatty acids were also identified as important for these time points. Bulbs samples harvested in the full flowering time point or after flowering grouped together in the PLS-DA analysis (Figure 8a). Signals highly correlated to these time points belonged to the sugars glucose and sucrose. The last time point (E: senescence) bulb samples were quite clearly separated from the others in the PLS-DA score scatter plot. Here the signals most important for this discrimination belonged to 4-HPP. Also correlated to this time point were the dd signals at δ 2.81 and 2.82 of asparagine and aspartic acid, and alkaloids signals of galanthamine, narciclasine, and citric acid. An unidentified compound (possibly kaempferol analogue) with doublet signals at δ 8.00 and δ 5.96 (J=8.3) was also important for the discrimination of this group.

^{1}H NMR signals correlated to the first two time-points in the leaves were identified by inspection of the PLS-DA loading scatter plot (Figure 8) and VIP plot. These signals belonged to galanthamine and narciclasine and the alkaloid precursor 4-HPP. Additional signals correlated to the earlier time point samples were unsaturated fatty acid signals, aspartic acid, alanine, fructose and glucose. Signals tentatively assigned to a kaempferol analogue (δ 5.96) and a malic acid conjugate were also important for the early time-point samples. Samples of the full flowering and after flowering time-points were quite similar, with the same ^{1}H NMR signals important for their discrimination. These included signals of secondary metabolites, such as chlorogenic acid, 5-*O*-feruloylquinic acid and rutin. Signals from flavonoids tentatively assigned to a quercetin analogue were also important for the discrimination. The senescent leaves of the last time point had metabolite profiles similar to the after flowering samples, except with no alkaloid signals present. Many of the other secondary metabolite signals (flavonoids, chlorogenic acids) were still present but at lower intensities. In the PLS-DA loading scatter plot signals assigned to *cis*-aconitic acid and malic acid was correlated to the last time point samples. Several unassigned signals were also seen to be important for the discrimination, such as a doublet at δ 5.60 (anomeric H of glycoside) and singlets at δ 1.15, 1.05 and 0.99.

In the root samples, the first four time points were mainly correlated to sugar signals. Signals of sucrose and fructose were important for discrimination of the first two time points, and those of glucose and arabinose for the flowering and after flowering points. Additional signals correlated to the first two and following two time points were those assigned to trigonelline and malic acid, respectively. The senescence root samples were

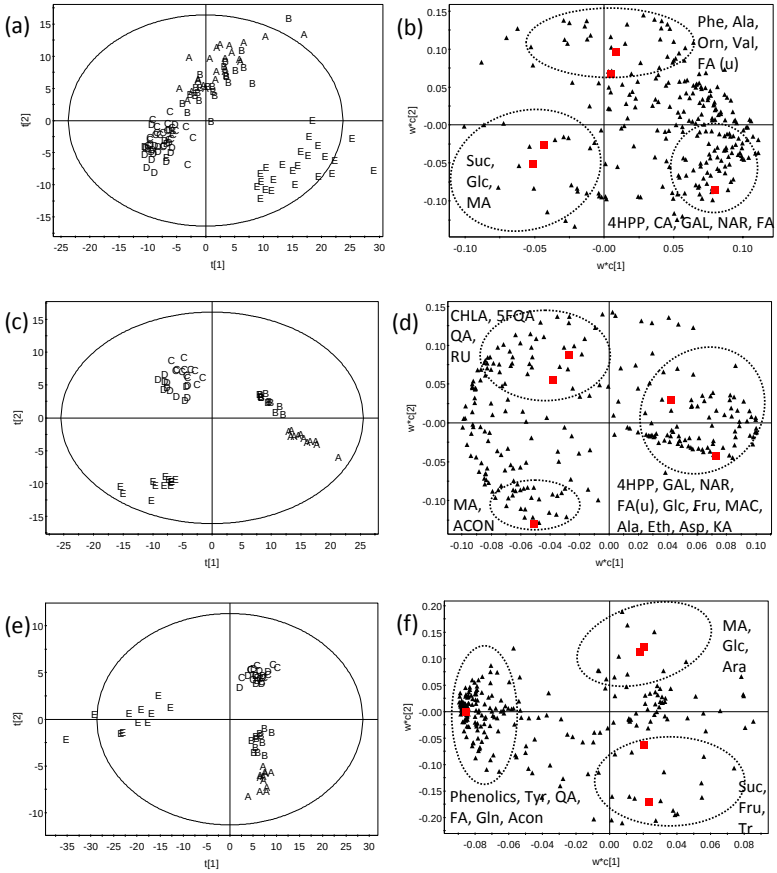


Figure 8. Score plots (PLS component 1 and 2) of PLS-DA results obtained from ^{1}H NMR spectra of *Narcissus pseudonarcissus* bulbs (a), leaves (c) and roots (e) and corresponding loading plots for bulbs (b), leaves (d) and roots (f). Samples assigned to classes according to time of harvest: A: shoots emerge, B: before flowering, C: full flowering, D: after flowering, E: shoot senescence. In the loading plots the squares (■) indicate the average of the classes; triangles (▲) represent ^{1}H NMR signal buckets. Signal buckets important for discrimination of the assigned classes are labeled; Suc: sucrose, Glc: glucose, Fru: fructose, Ara: Arabinose, MA: malic acid, MAC: Malic acid conjugate, Phe: phenylalanine, Ala: alanine, Orn: ornithine, Val: valine, FA: fatty acids, FA(u): unsaturated fatty acids, 4HPP: 4-hydroxyphenylpyruvate, CA: citric acid, GAL: galanthamine, NAR: narciclasine, CHLA: chlorogenic acid, 5FQA: 5-O-feruloyquinic acid, QA: Quercetin analogue, KA: kaempferol analogue, RU: rutin, Acon: cis-aconitic acid, Eth: ethanolamine, Asp: aspartic acid, Tyr: tyrosine, Tr: trigonelline.

characterized by more intense signals in the aromatic region of the spectra, and less intense sugar signals. The PLS-DA analysis revealed signals important for the discrimination of these samples belonging to tyrosine and various unidentified phenolic compounds. Signals of a quercetin analogue and *cis*-aconitic acid were also correlated to the senescence root samples, as well as signals assigned to fatty acids and glutamine.

Discussion

Besides the alkaloids, seasonal changes in the overall metabolite profiles of *Narcissus pseudonarcissus* cv. Carlton has to our knowledge not been reported before. In this study ^{1}H NMR-based metabolite profiling allowed changes in major primary and secondary metabolites to be observed in the bulbs, leaves and roots of the plant. The most notable changes in the bulb samples over the growing season were in the sugars, fatty acids and amino acids. In the leaves, the profile of aromatic compounds changed substantially throughout the season. Apart from clear changes in the alkaloid levels, there were also qualitative changes in the flavonoid and chlorogenic acids profiles. Rutin, the most abundant flavonoid glycoside, and other quercetin analogues increased around the time of flowering to reach maximum levels at or after flowering. In contrast, kaempferol analogues were higher in the early time points shortly after the shoots emerged from the soil. Chlorogenic acid and the related compound 5-*O*-feruloylquinic acid also peaked in the leaves at the time of flowering. Many roles have been attributed to flavonoids in plant development, such as in defense, allelopathy, hormone transport and regulation, plant architecture and modulation of reactive oxygen species (reviewed in Buer et al., 2010). Similarly, chlorogenic acid and related compounds are believed to be involved in stress responses, photoprotection, cell wall building and organogenesis (Grace et al., 1998; Franklin and Dias, 2011). As yet not much is known about the role of these compounds in geophytes such as *Narcissus*, but in this study seasonal changes occurred that seem related to the flowering phase. ^{1}H NMR spectra of the roots revealed the presence of trigonelline in the early time point samples. Other major changes in the root samples were the appearance of large phenolic signals at leaf senescence, possibly related to structural changes of the root cell walls before dormancy (Wilson and Anderson 1979). A large increase in fatty acids was also seen in the roots towards the end of the season. Such an increase may be related to increased deposition of the poly-aliphatic domain of suberin at this point in the season, as observed in onion roots by Meyer et al., (2011).

The alkaloid precursors tyrosine and 4-HPP were present in all time-points in the bulbs, with 4-HPP being highest in the last time point according to the PLS-DA. This suggests

that the alkaloids are being produced in the bulbs throughout the season. Higher levels of alkaloids in the leaves (before flowering) were correlated with higher levels of the alkaloid precursor 4-HPP. The root samples had similar levels of the alkaloids at all time-points, with the exception of the last one where the average concentration of haemanthamine increased. This was correlated to an increase in tyrosine, but not 4-HPP. No 4-HPP was detected in any of the root samples, suggesting that the increase in haemanthamine seen at the end of the season was the result of transport from other organs as opposed to biosynthesis in the roots. The increased tyrosine was likely related to the up-regulation of the phenylpropanoid pathway for biosynthesis of other phenolic compounds.

Quantitation of galanthamine and haemanthamine in the different organs during the growth season yielded results comparable to those of previous reports in cv. Carlton (Kreh 2002). The method used in this study also allowed quantitative analysis of narciclasine. In the bulbs, the narciclasine concentration reached a maximum before flowering, followed by a decrease towards the end of the season. In the leaves narciclasine was highest at the beginning of the season, with concentrations steadily decreasing during the season, similar to galanthamine and haemanthamine. Narciclasine was not detected in the root samples. Narciclasine was previously reported in Carlton bulbs at 100 mg/kg in fresh bulbs (Piozzi et al., 1969). Here we analyzed narciclasine content by dry weight, with comparable results. Compared to galanthamine, the haemanthamine and narciclasine concentrations in the bulbs are relatively low throughout the growing season. For extraction of a compound for industrial use, as high a concentration of the compound as possible is desired. However, if the bulbs are already being harvested for extraction of galanthamine, narciclasine can be an additional product obtained from the bulb material. Similarly, haemanthamine could also be obtained as a useful side-product from the bulbs. Making use of the basic properties of galanthamine and haemanthamine versus the acidic properties of narciclasine, these compounds can be separated relatively easily for further purification.

Early in the growth season, the leaves had relatively high concentrations of galanthamine, narciclasine and especially haemanthamine. Although higher concentrations of alkaloids could be obtained from the leaves harvested at this time than from the bulbs, the total amount of biomass and thus total yield of alkaloids are not very high at this time (Kreh 2002). Harvesting the whole plant for extraction of the bulbs and foliage at an early time point would be possible; however bulbs of a higher mass would be obtained at the end of the season at the usual harvest time. Also, for sustainable production of *Narcissus* plants for alkaloid extraction, harvesting bulbs at the normal time (as for ornamental plant production) would mean that some bulbs could be replanted for the next season, while the rest are extracted for the target compounds.

Narcissus pseudonarcissus cv. Carlton is already a source of galanthamine, and can also be used as a source of haemanthamine and/or narciclasine. ^{1}H NMR is a useful tool for investigating narciclasine and the other Amaryllidaceae alkaloids simultaneously.

Chapter 9. Tulip gum as novel source of 6-Tuliposide B

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Abstract

Certain tulip cultivars produce large amounts of gum in the bulbs when infected with the fungus, *Fusarium oxysporium*. Gummosis can also be induced by applying ethylene gas or ethylene-releasing substances to the bulbs after harvest. Previously, the composition of tulip gum has been studied in terms of large macromolecules. Many studies on the composition of tulip gum reported it to consist mainly of polysaccharides. The gum polysaccharides have been analyzed to determine sugar composition and molecular mass. Up to now relatively little is known about the gum in terms of low molecular weight metabolite content. In the first part of this chapter extracts of tulip bulb gum were analyzed by ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) spectroscopy and were found to contain 6-tuliposide B. This was the first time tuliposides were observed in the gum from tulip bulbs. Isolated tulipalins and tuliposides possess various bioactivities, such as antibacterial, antifungal and insecticidal properties. The presence of these bioactive molecules in tulip gum may suggest a protective role for this physiological response. The fact that this compound occurs in the gum in relatively pure form, means that tulip gum may be a good natural source of 6-tuliposide B and related compounds for industrial uses. In the second part of the chapter experiments are described on tulip bulbs of different cultivars to determine how various factors (ethylene and methyl jasmonate application, temperature, mechanical wounding and time) affect the gum production and tuliposide concentration in the gum. For quantitative analysis of 6-tuliposide B in the gum a quantitative ^{1}H NMR method was developed. The results show it is possible to induce tulip bulbs to produce large amounts of gum, containing 6-tuliposide B. With careful optimization of induction conditions, this method has potential for use as a production method for 6-tuliposide B as an industrial product.

Tuliposides in Tulip Gum

Introduction

The plant pathogenic fungus, *Fusarium oxysporum* causes major problems in the cultivation of tulips. Infection with this pathogen can cause severe losses, especially during storage. Infected bulbs release large amounts of ethylene gas, which can have negative effects on otherwise healthy bulbs. Such effects include flower abortion, poor rooting and excessive splitting (Kamerbeek and De Munk, 1976). Another unwanted effect is gummosis, the production of a gum-like substance that is released into the spaces between bulb scales. When a large amount of the gum is produced it can form blisters under the surface of the bulbs or be extruded to the outside (Figure 1). Gummosis has also been reported in stems of tulips (Saniewski et al., 1998).

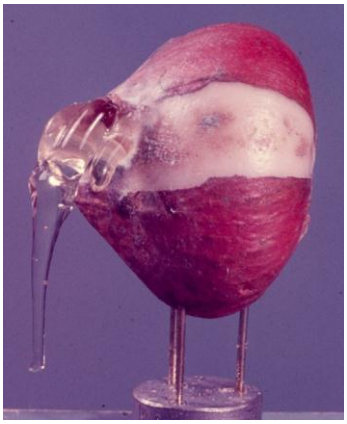

Figure 1. Gummosis in tulip bulb infected with *Fusarium oxysporum* (photo: PPO, Lisse).

Gummosis in tulips has been studied to better understand factors that induce the process, and the underlying carbohydrate metabolism involved (Saniewski et al., 2007). The composition of tulip gum has been mostly studied in terms of large macromolecules. Polysaccharides from tulip gum induced on tulip stems have been analyzed to determine sugar composition (De Munk and Saniewski, 1989) and molecular mass (Skrzypek et al., 2005). These studies showed that tulip (stem) gums consist of glucuronoarabinoxylan with an average molecular weight of ca. 700 kDa. Up to now relatively little is known about the gum in terms of small (low molecular weight) metabolite content. In this study tulip gum was analyzed by ^{1}H NMR spectroscopy to

investigate its low molecular weight metabolite profile. The aim was to determine whether the gum contained any interesting or novel small metabolites.

Materials and methods

Tulip gum (50 mg) from cultivar Apeldoorn was extracted with 1.5 mL MeOD and KH_2PO_4 buffer (pH 6), 1:1 (Kim et al., 2010) by vortexing for 30 s and sonication for 30 minutes. The sample was centrifuged and 800 µL of the solvent was collected for ^1H NMR analysis. Additional tulip gum samples were collected from bulbs of cultivar Madame Lefeber, Apeldoorn and Yokohama. These samples were extracted in the same way as described and analyzed by ^1H NMR. Tulip bulbs of two different cultivars (Santander and Flair) were ground to a fine powder in liquid nitrogen. The ground bulb material was freeze-dried and once dry, 50 mg was weighed into 2 mL eppendorf tubes. The bulb material was extracted in the same way as the tulip gum described above (KH_2PO_4 buffer in D2O -MeOD 1:1)

Results

To investigate the content of small metabolites in tulip gum, a gum sample collected from a bulb of cultivar Apeldoorn was extracted with a standard extraction solvent used in NMR-based plant metabolite analysis (methanol/phosphate buffer, 1:1)(Kim et al., 2010). Inspection of the NMR spectra revealed some high intensity signals in an otherwise relatively clean spectrum (Figure 2). Characteristic doublet signals at δ 5.19 and δ 4.59 indicated the presence of the anomeric protons of alpha and beta glucose moieties. Other interesting features were singlets at δ 5.98 and δ 5.75. Further 2D NMR experiments allowed assignment of signals and identification of this compound as 6-tuliposide B (Figure 3 and 4). Signal assignments are shown in Table 1, and corresponds well to those reported in the literature (Shoji Kazuaki et al., 2005),(Christensen and Kristiansen, 1999) , with small differences in chemical shift values due to the use of a different NMR solvent. Integrals from the anomeric proton (H-1: δ 5.19 and δ 4.59) showed that the equilibrium between the α- and β-forms of 6-tuliposide B was approximately 4:6 in this solvent.

Tulip bulbs of two different cultivars were extracted in the same way as the tulip gum (the KH_2PO_4 buffer-MeOD, 1:1) to compare the compositions of the extracts. Bulbs of cultivar Santander and Flair were compared with tulip gum from cultivar Madame Lefeber. As with tulip gum from cultivar Apeldoorn, the extract of the Madame Lefeber gum was found to contain 6-tuliposide B in a relatively pure state. The 6-tuliposide B signals were also seen in the ^1H NMR spectra of the bulb extracts (Figure 5b and c), but at about 25% of the intensity as compared to the gum signals. The bulb extracts were

more complex and contained signals characteristic of sugars, amino acids and fatty acids, and some phenolic compounds (Choi et al., 2006; Abdel-Farid et al., 2007; Kim and Verpoorte, 2010). The bulb of cultivar Santander had some prominent signals at δ 5.79 and δ 6.30, in the same region as the tuliposide B signals. A prominent triplet at δ 2.57 could also be seen with an integrated area double that of each of the other two signals. These and other signals belonging to the sugar moiety matches signals previously reported for tuliposide A in the literature (Christensen, 1995).

Table 1. Assigned ^{1}H NMR signals of 6-tuliposide B extracted from tulip gum, in MeOD and KH_2PO_4 buffer (pH 6) 1:1, measured at 500 MHz.

H	**δ (ppm)**	**Splitting pattern**	**J (Hz)**	**integral**	**COSY**
3'a	5.98	s		1	
3'b	5.75	s		1	2.92, 2.75
4'	3.83	dd	4.35, 8.05	1	2.92, 2.76
5'a	2.92	dd	14.8, 4.35	1	2.76, 3.83, 5.75
5'b	2.76	dd	14.8, 8.05	1	2.92, 3.83, 5.75
β-glucose:					
1	4.59	d	8.2	0.6	3.20
2	3.20	dd	8.8, 8.2		4.59, 3.44
3	3.44	t	8.86		3.20
4	3.52	d	11.86		3.70
5	3.70	m			3.52, 3.88, 4.03
6a	3.88	dd	12.3, 2.0		4.03, 3.70
6b	4.03	dd	12.3, 2.0		3.88, 3.70
α-glucose:					
1	5.19	d	3.75	0.4	3.48
2	3.48	dd	3.75, 9.8		5.19,
3	3.63	t	9.8		3.24
4	3.24	t	9.8		3.63
5	3.48	m			3.73, 3.69
6a	3.73	dd	12.0, 5.0		3.69
6b	3.69	dd	12.0, 2.0		3.73

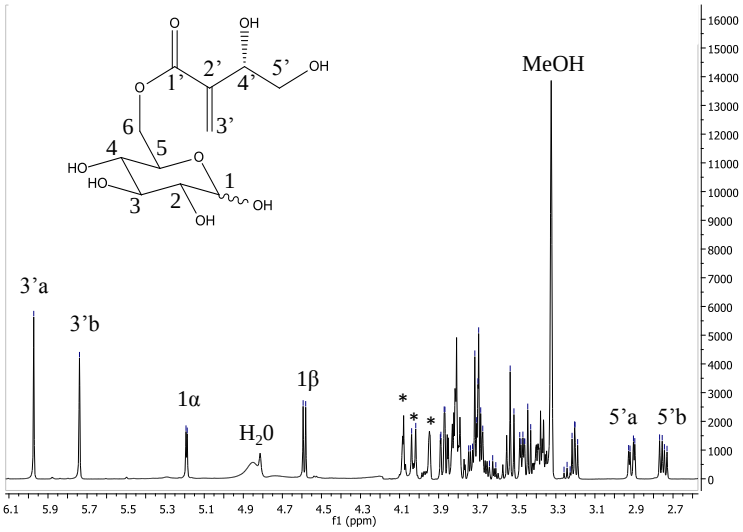

Figure 2. Structure of 6-tuliposide B and ^{1}HNMR spectrum (500 MHz) of tulip gum in MeOD and KH_2PO_4 buffer (pH 6) 1:1, with non-overlapping signals labeled. Signals not belonging to 6-tuliposide B are labeled with an asterisk (*).

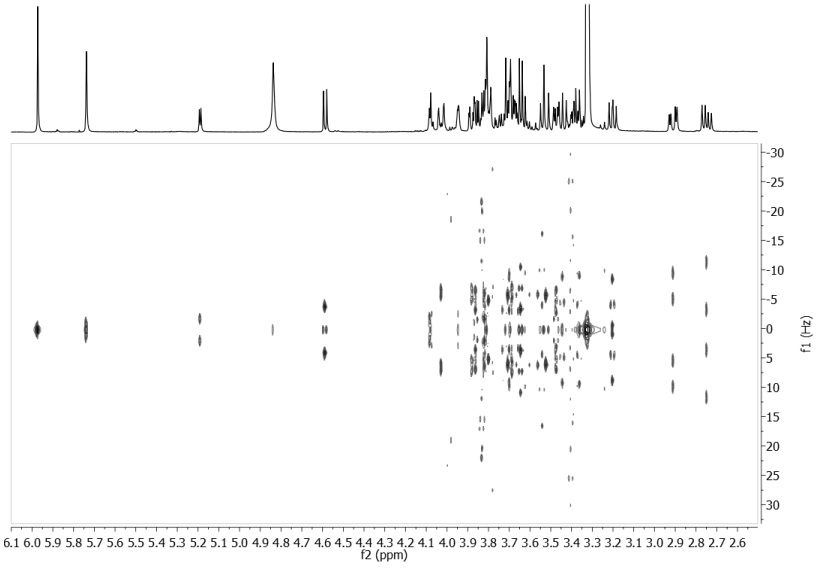

Figure 3. 2D J-Resolved spectrum of 6-tuliposide B recorded in MeOD and KH_2PO_4 buffer (pH 6) 1:1 at 500 MHz.

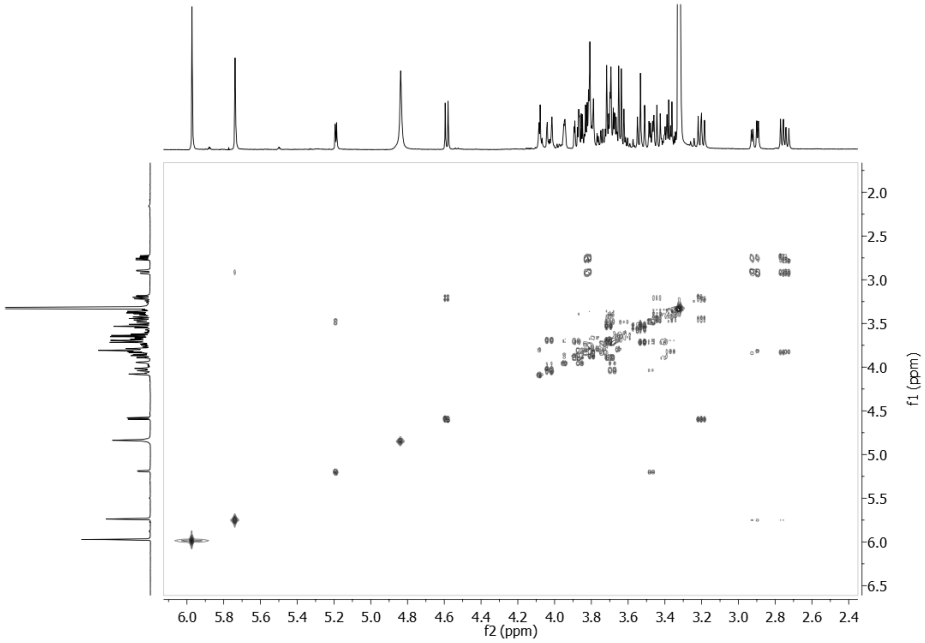

Figure 4. 2D COSY spectrum of 6-tuliposide B recorded in MeOD and KH_2PO_4 buffer (pH 6) 1:1 at 500 MHz.

The color of the tulip gum was seen to vary from clear and yellowish to opaque and brownish. Gum samples of different colors were extracted and analyzed by ^{1}H NMR to investigate their content. Two clear gum samples collected from two different cultivars (Apeldoorn and Yokohama) were analyzed. A brownish sample collected from inside a blister on the outside of an Apeldoorn bulb was also analyzed. The 6-tuliposide B was present in all three samples, with the Yokohama gum sample containing the smallest amount (Figure 6). In the clear Apeldoorn gum 6-tuliposide B was present in a relatively pure state. In contrast the brown Apeldoorn gum from the blister also contained the compound tentatively identified as tuliposide A. Two triplet signals at δ 6.19 and δ 5.82 could be seen in this sample as well. These signals are most likely due to the presence of tulipalin A (Tschesche et al., 1968, 1969; Christensen Lars P, 1999). In the aromatic region of the spectrum there are also signals that probably belong to phenolic compounds. These compounds may be responsible for the brownish color, as these signals are absent in the clear gums.

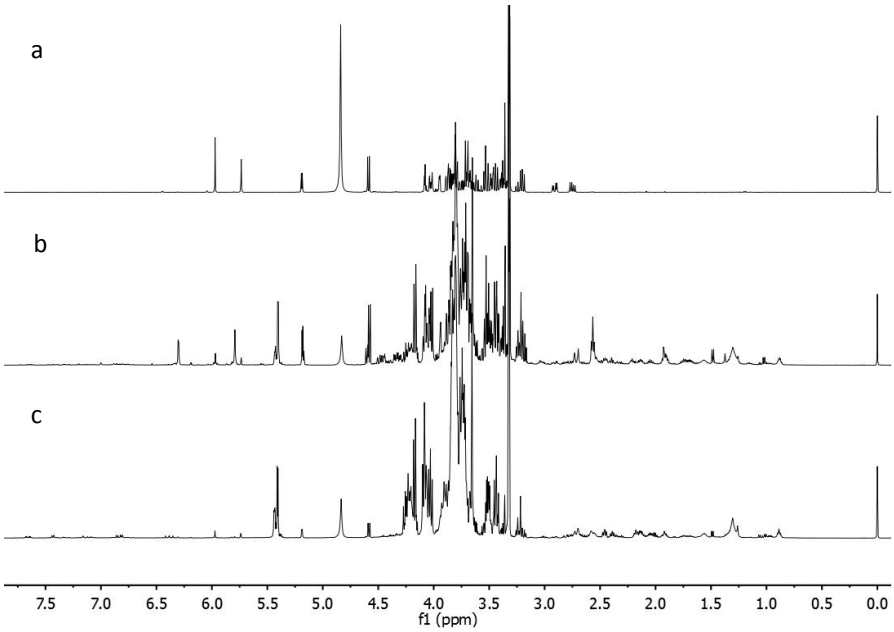

Figure 5. ^{1}H NMR spectra of extracts of tulip gum (a) and bulbs of cultivar Santander (b) and Flair (c) showing the differences in intensity of the 6-tuliposide B signals at δ 5.98 and δ 5.75. ^{1}H NMR spectra recorded in MeOD and KH_2PO_4 buffer (pH 6) 1:1 at 500 MHz.

Discussion

Tuliposides and their related α-methylene-γ-butyrolactones, tulipalins, are known to occur in various organs of the tulip plant. The presence of tuliposides or tulipalins in tulip gum has not been reported before. Studies on the composition of tulip gum in the past focused on the molecular weight of the gum polysaccharides (Skrzypek et al., 2005), and the sugar composition of the polysaccharides (De Munk and Saniewski, 1989). These analyses usually involved hydrolyzing the gum samples for several hours in acid, conditions which would likely have caused degradation of the tuliposides.

Six tuliposides and two tulipalins have been isolated from tulips. The structures of these are shown in Figure 7. Tuliposides A and B are widely distributed in the genus *Tulipa*, with different ratios of the different kinds being reported. Tulips in section Leiostemones usually contain larger amount of tuliposide B than A, while those in section Eriostemones have large amounts of both A and B with no consistent interrelationship between the two (Christensen and Kristiansen, 1999). Tuliposide D

occurs in large amounts in *T. patens*, and only in trace amounts in other species (Christensen and Kristiansen, 1999).

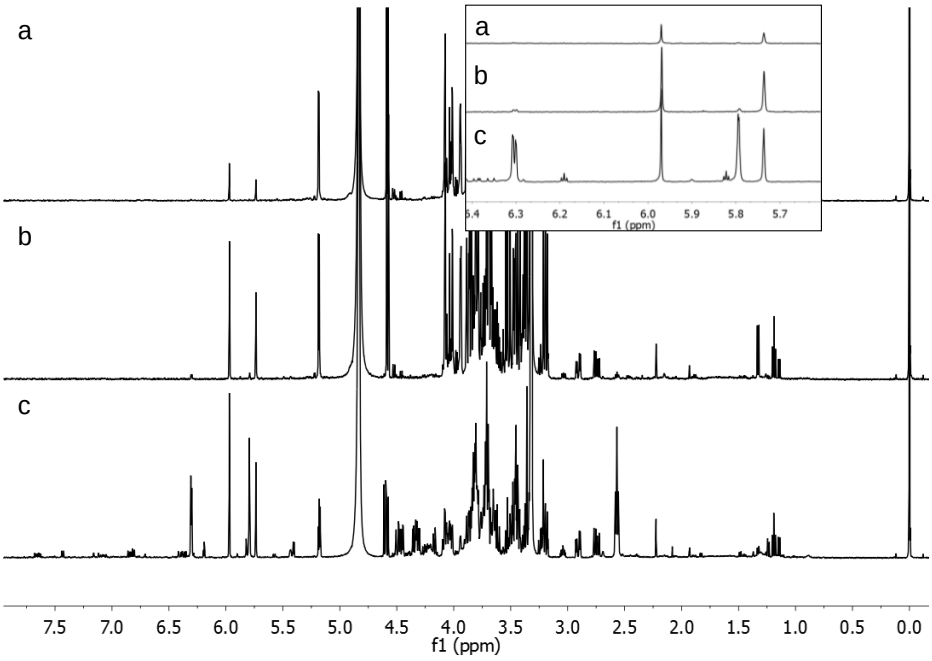

Figure 6. ^{1}H NMR spectra of extracts prepared from clear Apeldoorn gum (a), clear Yokohama gum (b) and brown Apeldoorn gum. ^{1}H NMR spectra recorded in MeOD-buffer at 500 MHz. Insert shows magnified region of spectra from about δ 6.45 to δ 5.65.

Tuliposide F was first isolated from *T. turkestania* and has also been found in all taxa investigated since. The name Tuliposide C was given to a compound from tulip in an early study, but since this compound was never fully characterized, the next tuliposide that was completely elucidated was named tuliposide D.

Tulipalins are released from tuliposides spontaneously in response to increased pH or enzymatically (Beijersbergen and Lemmers, 1972). Above pH 5.2 glucose is released from tuliposide, and the free acids lactonize to form the tulipalins (Schönbeck and Schroeder, 1972). Above pH 7.0 the free acids are reportedly present. Tulipalin A was initially isolated from tulip bulb material as an antifungal compound (Tschesche et al., 1968). The precursor tuliposide A was isolated thereafter as well as tulipalin B and its

precursor tuliposide B. It was first believed that tuliposides store tulipalins for release only after infection or mechanical damage, but it was later shown that free tulipalins also occur at low concentrations in healthy plants. Tuliposide A and tulipalin A have allergenic properties, and are responsible for the skin irritation often suffered by tulip harvesters (called “tulip fingers”) (Hausen et al., 1983; Gette and Marks, 1990). Tulipalin B and the other tuliposides are not allergenic to humans, but these compounds appear to play a protective function to the plant (Beijersbergen and Lemmers, 1972; Schönbeck and Schroeder, 1972).

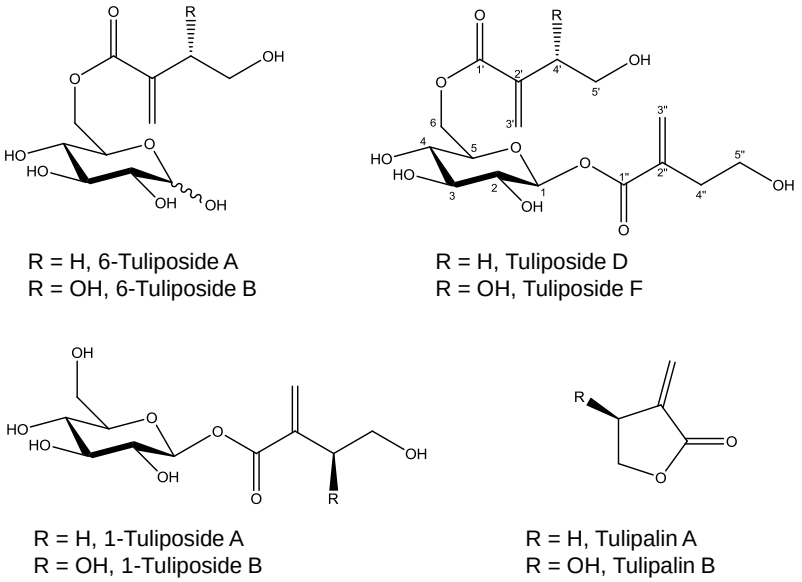

Figure 7. Chemical structures of tuliposides and tulipalins.

Isolated tuliposides and tulipalins have been tested for various bioactivities. As mentioned already, tulipalin A was found to be active against *Fusarium oxysporum* f.sp. *tulipae* (Bergman and Beijersbergen, 1968). The presence of tuliposide A seems to protect against fungal infection of bulbs by *F.oxysporum* and *Botrytis cinerea*, but this may be due to the release of tulipalin A upon infection (Schönbeck and Schroeder, 1972). Both 1-tuliposide A and B were found to have antibacterial properties against *Bacillus subtilis* (Tschesche et al., 1968). Activity against *Pythium debaryanum* was also reported for 1-tuliposide B (Tschesche et al., 1969). In a more recent study, 6-tuliposide B was tested for antimicrobial activity against various bacterial strains. Activity was found in all bacteria tested (including gram-positive and –negative types),

namely *Escherischia coli, Salmonella enteritidis, Pseudomonas aeruginosa, P. glume, P. avenae, Staphylococcus aureus* and *Bacillus subtilis*. Tulipalin B was reported to have antibacterial activity against *E.coli* (Lee et al., 2008). The very effective antimicrobial properties of these compounds are reflected in a patent submitted recently (Park et al., 2008). In addition to the antimicrobial activities of the tulipalins and tuliposides, insecticidal activity has also been reported for tulipalin A against thrips (*Thrips palmi, Frankliniella occidentalis, F.intonsa*) and mite (*Tetranchus urticae*) (Kim et al., 1998).

Gum formation in plants is generally thought to be a defensive response against pathogen infection, to stop their spread and isolate infected tissue (Boothby, 1983). In tulips, microscopic analysis of gums induced by *F. oxysporum* showed the presence of hyphae and spores of the pathogen in the gum (Saniewska et al., 2004). In another study gum induced by *F. oxysporum* stimulated mycelial growth and spore formation of the fungus when added to its culture media (Saniewska, 2002a,b). This effect was seen not only on the specialized tulip pathogen, but also in cultures of *F. oxysporum* f.sp. *callistephi* and *narcissi* (Saniewska, 2002c). From these results it was concluded that the gum does not inhibit the development of the fungus, and that it may stimulate mycelium growth or can be used as a substrate by the fungus (Saniewska et al., 2004).

The discovery of tuliposides in tulip gum raises further questions about the gum's role in the plant's response to pathogen attack. Tulipalin A (Figure 7) is reported to be toxic to *F. oxysporum*, while tuliposide A is not. Neither Tuliposide B nor tulipalin B is toxic to *F. oxysporum narcissi* (Beijersbergen and Lemmers, 1972). *In vitro* studies have reported strong antibacterial activity of 6-tuliposide B and tulipalin B (Beijersbergen and Lemmers, 1972; Lee et al., 2008; Shigetomi et al., 2010). Antifungal activity has also been demonstrated against plant pathogen *Botrytis cinerea*, although the specialized tulip pathogen *Botrytis tulipae* has evolved ways to inactivate the toxic compound (Schönbeck and Schroeder, 1972).
Tuliposides are active against various microorganisms and their function in the plant is believed to be protection against pathogens above and below ground (Beijersbergen and Lemmers, 1972; Schönbeck and Schroeder, 1972). Tuliposides are here reported in tulip gum for the first time. It is possible that they also serve a protective role, with the gum acting as a barrier to pathogens and the tuliposides providing further chemical defense. This barrier may not be effective against *F. oxysporum*, but could help protect against infection by bacteria and other fungi. This may be a case of a specialized fungal strain evolving ways to evade the host plant's defense response, as in *Botrytis tulipae* and tulips (Schönbeck and Schroeder, 1972).
Not all tulip cultivars can be induced to produce gum (Kamerbeek et al., 1971). It is not known whether gummosis occurs in wild species of tulips. The possibility exists that

gummosis is an excessive hyper-sensitive response to ethylene that has been unintentionally selected for in certain cultivars through centuries of breeding. The presence of tuliposides in the gum could then be due to the compounds being in the cells of the tissue where gum formation takes place.

The role of the gum and the metabolites it contains may not be completely understood. The presence of one or two tuliposides in relatively pure form in gum extracts, however, makes this a potentially interesting source of these bioactive compounds. Compared to isolating tuliposides from tulip bulbs or leaves, their isolation from tulip gum presents a much simpler task. There is interest in obtaining tuliposides in pure form from plant material (Damude et al., 2003; Shigetomi et al., 2008). Not only the tuliposides, but also the tulipalins have properties that make them potentially interesting industrial compounds (Pickett and Q. Ye, 2008). Gummosis can be induced in otherwise healthy tulip bulbs by exposing them to ethylene or ethylene-releasing substances (De Munk and Saniewski, 1989). This could potentially be done on large scale for the isolation of tuliposides (and tulipalins) for industrial use.

Studies on gum induction in various tulip cultivars.

Introduction

In ornamental tulip cultivation, infection with the pathogenic fungus *Fusarium oxysporum* f.sp. *tulipae* causes many problems. Infection of the bulbs results in the production of huge amounts of ethylene by the fungus (Saniewska et al., 2005). Ethylene is a plant hormone and its presence in excessive amounts can cause physiological effects such as flower abortion, poor rooting, excessive splitting and gummosis in tulips (Kamerbeek and De Munk, 1976). These problems lead to severe losses of infected bulbs, particularly during storage. Bulbs not directly infected by the pathogenic fungus can also be affected, as the excessive ethylene produced in infected bulbs can cause the previously mentioned effects when released into the storage area.

Gummosis (the accumulation and exudation of gum) does not occur in all tulip cultivars, as they are not all equally sensitive to ethylene. In sensitive cultivars, however, gummosis can be triggered by ethylene concentrations as low as 0.1 ppm. The extent of gummosis has been reported to increase with increasing ethylene concentration, up to about 20 ppm (Kamerbeek et al., 1971). It is also known that in cultivars where gummosis occurs, the sensitivity to ethylene varies over time. Sensitivity to ethylene is reportedly highest in the first four weeks after harvest, after which it decreases (Kamerbeek et al., 1971).

Methyl jasmonate (JA-Me) and related compounds (jasmonates) play an important role in the signal transduction pathway in plants in response to stresses (Koiwa et al., 1997). Jasmonates are known to induce gum formation in various plant species. In tulip stems, jasmonates have been shown to play an essential role for gum formation. JA-Me has been shown to alter sugar metabolism in tulip stems, with some stem carbohydrates likely being channeled to gum formation (Skrzypek et al., 2005). While the exact mechanism of ethylene in stimulating gum formation is not entirely clear, it appears that interactions between ethylene and JA-Me signal transduction pathways occur.

The gum of tulip bulbs was shown to contain the small molecule 6-tuliposide B. This compound and related compounds has some interesting bioactivities and properties, as discussed in the previous section. The fact that this compound occurs in the gum in relatively pure form, means that tulip gum may be a good natural source of 6-tuliposide B for industrial uses. Otherwise healthy tulip bulbs can be induced to produce gum, potentially providing a source of the raw material for tuliposide extraction. In this way gummosis, usually seen as a problem in the ornamental tulip industry, can potentially be turned into a solution for a sustainable supply of a useful bioactive metabolite.

In this study experiments were conducted on tulip bulbs of different cultivars to determine how various factors (ethylene and JA-Me application, temperature, mechanical wounding and time) affect the gum production and tuliposide concentration in the gum. For quantitative analysis of 6-tuliposide B and related compounds in the gum a quantitative ^{1}H NMR method was developed. The aim was to see whether production of tulip gum can be optimized as a potential source 6-tuliposide B.

Materials and methods

Development of qNMR method

Different polar NMR solvents were tested as extraction solvent for tulip gum. Solutions of MeOD, D_2O, KH_2PO_4 buffer (pH 6) and MeOD-Buffer (1:1, v/v), were prepared containing maleic acid as internal standard at 0.1 mg/mL. Tulip gum samples (50 mg of freeze-dried Madame Lefeber gum) were extracted with 1.5 mL of each solvent by vortexing for 20 s, then sonication for 30 minutes. The samples were centrifuged and the supernatant was collected for ^{1}H NMR analysis. To assess the extraction recovery of tuliposide B from the gum, a freeze-dried Madame Lefeber gum sample (56 mg) was extracted three times with 1.5 mL buffer as described above. After each round of extraction the supernatant was collected and measured in ^{1}H NMR. The solubility of tulip gum in buffer was tested by adding 1 mL of buffer to 2, 5 and 12 mg samples of freeze-dried Madame Lefeber gum. The samples were sonicated for 10 minutes,

vortexed and left overnight. The next day samples were inspected to assess dissolution of the gum. The samples were centrifuged, and aliquots of 800 µL were collected for ^{1}H NMR analysis.

Extraction method for ^{1}H NMR quantitation of tuliposide B

Gum samples were freeze-dried and ground to a fine powder with a laboratory blender or by pestle and mortar, depending on the available amount. Five mg of freeze-dried ground gum was weighed into 2 mL microtubes. To each gum sample was added 1 mL of KH_2PO_4 buffer (pH 6) containing 0.01 TMSP and 0.1 mg/mL maleic acid. After vortexing for 30 s and ultrasonicating for 15 min, samples were left overnight in the dark at 4 °C to solubilize. The next day samples were vortexed again, then centrifuged for 5 min at 13 000 rpm. The supernatant (800 µL) was collected and transferred to NMR tubes. Samples were stored at 4 °C until measurement.

NMR measurements

^{1}H NMR spectra were recorded with a Bruker AV 500 spectrometer (Bruker, Karlsruhe, Germany). For each sample 64 scans were recorded using the following parameters: 0.167 Hz/point, pulse width (PW) 4.0 µs and relaxation delay (RD) = 1.5 s. FIDs were Fourier transformed with LB = 0.3 Hz. Manual phase adjustment and baseline correction were applied as well as calibration with internal standard TMSP to 0.0 ppm. The area under the maleic acid signal at 6.20 ppm was compared to the area under the singlet at 5.98 ppm of tuliposide B, and used to calculate the concentration of tuliposide B in the original gum sample. Two-dimensional J resolved NMR spectra and ^{1}H–^{1}H correlated spectroscopy (COSY) spectra were also recorded.

Bulb treatments and induction of gummosis

Bulbs of *Tulipa gesneriana* L. cv. Apeldoorn, cv. White dream and cv. Madame Lefeber (all sift size 10/12, harvested 12 July 2011) were used in these studies. For ethylene treatments, peeled bulbs were placed in a sealed chamber containing a known amount of ethylene gas, for one to three days. When bulbs were in the chambers for more than one day, the ethylene gas was replenished every 24 hours. Before and after ethylene treatment the bulbs were stored at 20 °C. In some treatments bulbs were mechanically damaged by pricking the bulbs with a push pin (four pricks around thickest part of bulb). Other mechanical damage treatments involved bruising the bulbs by pressing them with a flat hand, cutting the bulb from top to bottom four times (penetrating the first and second outer bulb scales) or cutting the bulbs into four segments (top to bottom). An experiment was carried out with methyl jasmonate (JA-Me) applications in combination with ethylene for gum induction. For these treatments

a special vat with a volume of 3.8 L was used. Ten bulbs were placed in each vat, and JA-Me was applied in one of two ways. The JA-Me was applied as a spray (7.6 or 76 µL in 1 mL water sprayed over the bulbs with a perfume sprayer) or on filter paper (7.6 or 76 µL applied to a piece of filter paper, folded and placed in the vat). The vats were sealed and after sealing ethylene (30 ppm) was added to the vat by means of a syringe through the lid septum. Vats were left for 24 hours at 28 °C, after which they were opened and stored at 20° for five days. Each treatment was carried out with 10 bulbs per category, and NMR measurements were carried out on one sample of each treatment representing the pooled gum.

Results

NMR method development for tuliposide quantitation

^{1}H NMR was found to be a suitable method to analyze the tulip gum extracts. The sharp singlet peaks of 6-tuliposide B in a relatively non-crowded region of the NMR spectrum also make it a suitable method for quantitative NMR analysis. For the development of a quantitative NMR (qNMR) method, an internal standard was needed, as well as a good extraction solvent. An internal standard should ideally give a signal close to the target signal to be used in quantitation. It should also not be overlapped with any other signal in the spectrum (Pauli et al., 2005). The internal standard and target compound should be soluble in the chosen NMR solvent, and the compound of interest should be extracted with a high recovery. The stability of the target compound in the NMR solvent is also important for accurate quantitation. Maleic acid was identified as a good candidate for use as an internal standard, as it gives rise to a singlet in the region of δ 6.20 – δ 6.32 in polar NMR solvents.

Different polar NMR solvents were tested for use as extraction solvent for tulip gum (Figure 8). In all the NMR solvents the maleic acid signals were close to the tuliposide singlet signals, but not overlapping with them or any other signals. Relative to maleic acid, the 6- tuliposide B signals were the most intense in the water and KH_2PO_4 buffer extracts, indicating that these are good extraction solvents for this target compound from the gum matrix. Even though D_2O seemed to extract slightly more 6-tuliposide B than the buffer, it was decided to proceed with the buffer as extraction solvent. It is known that tuliposides are unstable at pH above 7 (Beijersbergen and Lemmers, 1972), so it was decided to use an extraction solvent that controls the pH to keep the extract at a slightly acidic pH.

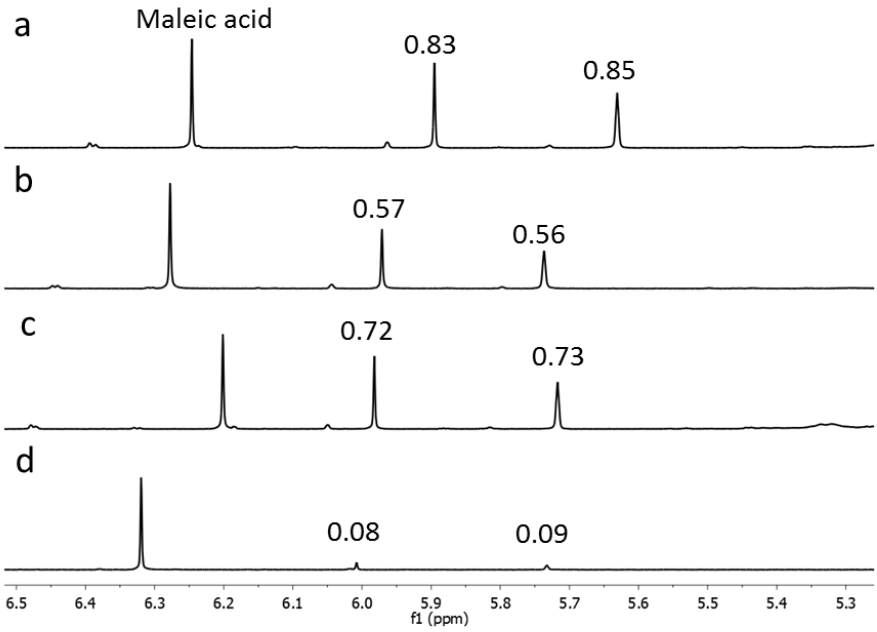

Figure 8. ^{1}H NMR spectra of tulip gum extracted with different polar solvents: (a) D_2O, (b) MeOD-KH_2PO_4 buffer, pH 6.0 (1:1), (c) KH_2PO_4 buffer, pH 6.0, (d) MeOD. The ratio of the 6-tuliposide B singlet signal integrals relative to the maleic acid signals are shown in the Figure.

The extraction recovery of 6-tuliposide B from the gum was tested. It was found that extracting 50 mg of tulip gum in 1.5 mL of KH_2PO_4 buffer (the weight and volume typically used in the NMR metabolomics method) does not give exhaustive extraction of the compound, even after more than three rounds of extraction. This indicated saturation of the solvent, or is related to the fact that the gum is not solubilized completely at these ratios. Smaller volumes of gum were dissolved in buffer to determine how much gum can be completely dissolved in the solvent. Table 2 shows the result of dissolving different amounts of the same gum sample in 1 mL of buffer, and using maleic acid to quantify the amount of 6-tuliposide B in each sample. The gum was completely dissolved when 2 or 5 mg was solubilized in 1 mL of buffer. The amount of 6-tuliposide B also increased proportionally with the amount of gum dissolved up to about 5 mg of gum. Samples prepared with more gum did not dissolve completely and the increase in tuliposide was not exactly proportional to the increase in the amount of gum. Therefore it was decided that 5 mg of gum dissolved per mL of buffer would be optimal for sample preparation.

Table 2. Increase in 6-tuliposide B signal (δ 5.98) in proportion to the amount of gum dissolved in 1 mL of KH_2PO_4 (pH 6.0) buffer.

Gum (mg DW)	Area ratio of 6-tuliposide B singlet to maleic acid singlet
2.0	0.14
5.0	0.36
12.0	0.27

Quantitation of 6-tuliposide B in gum from different cultivars

The presence of 6-tuliposide B was first discovered in gum from cultivar Apeldoorn. The process of gummosis is known to occur in certain tulip cultivars (Kamerbeek & De Munk 1976; Kamerbeek 1971). Bulbs of some cultivars observed to be sensitive to gummosis were obtained and induced to produce gum. The gum was analyzed to determine whether 6-tuliposide B occurs in all the samples. The ^{1}H NMR analyses showed the presence of 6-Tuliposide B in all the gum samples tested (Figure 9). The concentrations ranged from less than 10 mg/g to more than 90 mg/g DW. In cultivars where more than one gum sample was obtained, some variation can be seen between samples (e.g. Madame Lefeber). Since these gum samples were obtained at different times in different conditions, not much can be concluded about the concentrations of 6-tuliposide B in the gum.

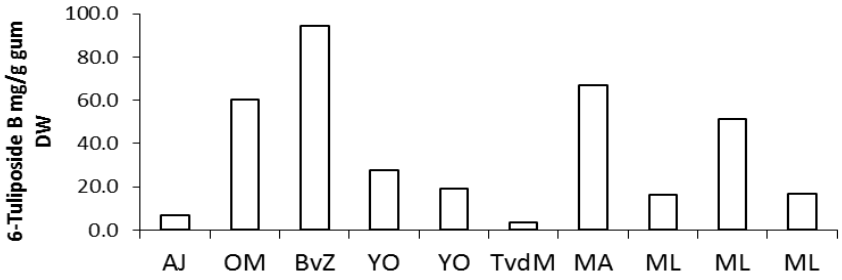

Figure 9. 6-Tuliposide B concentration in gum of different tulip cultivars. AJ: Anna Jose, OM: Orange Monarch, BvZ: Ben van Zanten, YO: Yokohama, TvdM: Tineke van der Meer, ML: Madame Lefeber.

Effect of mechanical injury on gum production and 6-tuliposide B in gum

The effect of mechanical injury on the 6-tuliposide B content in tulip gum in three different cultivars was investigated. Bulbs of cultivars White dream, Madame Lefeber and Apeldoorn were treated with ethylene for 24 hours to induce gummosis. Half the

bulbs of each cultivar were pricked with a needle, and the rest were not damaged. The concentration of 6-tuliposide B was determined and the results are shown in Figure 10. The White dream bulbs produced very little gum, and the tuliposide concentration was not determined in these samples. The Apeldoorn gum had a higher 6-tuliposide B concentration than the Madame Lefeber samples, but less gum was produced by the bulbs. In this experiment mechanical injury with a needle did not have a big effect on the gum production in the Madame Lefeber samples. In the Apeldoorn bulbs the 6-tuliposide B concentration in the damaged gum samples was lower than the control.

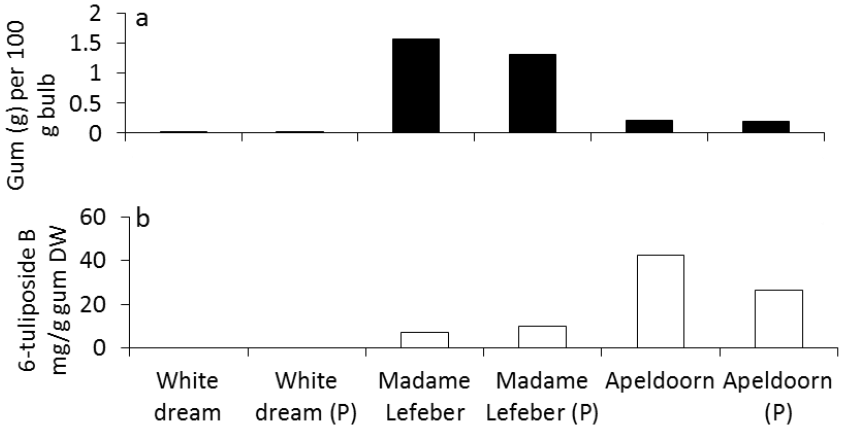

Figure 10. Weight of gum (a) and 6-tuliposide B concentration (b) of gum of three tulip cultivars, treated with ethylene for 24 hours and stored at 20°C for three days. WD: White dream, WD-P: White dream pricked, MD: Madame Lefeber, MD-P: Madame Lefeber pricked, AP: Apeldoorn, AP-P: Apeldoorn pricked.

Effect of duration of ethylene treatment on gum production and 6-tuliposide B in gum

The effect of the duration of ethylene treatments on tuliposide content in the gum was investigated in bulbs of cultivar Apeldoorn, White dream and Madame Lefeber. Bulbs were treated with ethylene for 24 or 48 hours, and then kept for three days at 20°C. Half the bulbs were mechanically damaged by pricking and half were untreated. The results are shown in Figure 11. In all the cultivars, more gum was produced with longer ethylene treatments. Mechanically damaging the bulbs by pricking seemed to enhance gum production in the Apeldoorn and Madame Lefeber bulbs, but not the White dream bulbs. 6-Tuliposide B concentrations were lower in gum of pricked bulbs than untreated bulbs. The undamaged Apeldoorn bulbs treated with ethylene for 24 hours had the

highest concentration of 6-tuliposide B (239 mg/g DW). A single treatment was carried out where bulbs of Apeldoorn were exposed to ethylene for a total of 168 hours. This resulted in the production of 6.48 g gum per 100 g bulbs, the highest amount in any of the experiments performed. The 6-tuliposide B concentration in the gum was relatively low, at 11.8 mg/g gum (DW).

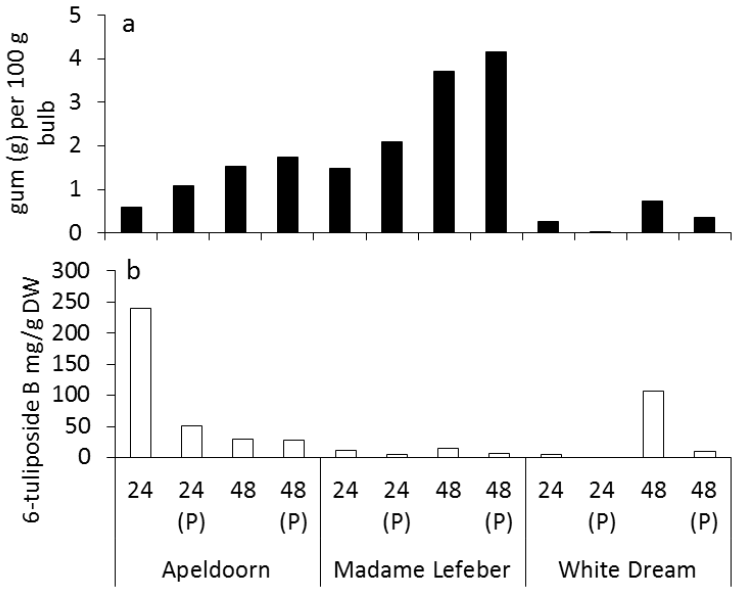

Figure 11. Weight of gum (a) and 6-tuliposide B concentration (b) of gum produced by Apeldoorn, Madame Lefeber and White Dream bulbs, induced with ethylene (Eth) for 24 or 48 hours. Some bulbs were mechanically damaged by pricking with a needle (P).

Effects of different types of mechanical damage on gum production and 6-tuliposide content

Bulbs of cultivar Apeldoorn were given different types of mechanical damage to determine if this had an effect on the content of 6-tuliposide B in the gum, and the amount of gum produced. Bulbs were either pricked with a needle as before, bruised by pressing down on them with a flat object, cut with a knife or quartered. For each treatment, bulbs were treated with ethylene for 24, 48 and 72 hours. The results of the 6-tuliposide B quantitation are shown in Figure 12. From Figure 12a can be seen that the duration of ethylene treatments had a clear effect on the amount of gum produced, with

it increasing with the duration of ethylene treatment. An exception is the quartering treatment, which resulted in very little gum production. The concentrations of 6-tuliposide B were highest in the 24 hour-treated samples in all treatments, and lower in the longer ethylene treatments. The control bulbs not mechanically damaged had the highest 6-tuliposide B concentration in all durations of ethylene treatment.

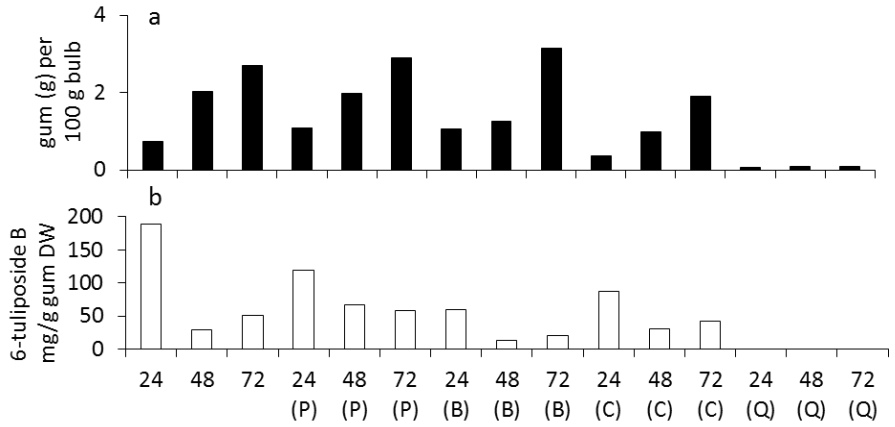

Figure 12. Weight of gum (a) and 6-tuliposide B concentration (b) of gum produced by Apeldoorn bulbs, induced with ethylene for 24, 48 or 72 hours and stored at 20°C for three days. Bulbs were mechanically damaged by pricking with a needle (P), bruising (B), cutting (C) or quartering (Q).

Effect of time after harvest on gum production and tuliposide content of tulip gum

In the previous investigations, there were big differences in the tuliposide content of gum that had been induced in the same way (e.g. 24 hour ethylene treatment, no mechanical damage in Figure 10 and Figure 11). The difference between the bulbs in those treatments was that they had been induced to produce gum at different lengths of time since being harvested. An experiment was performed with Apeldoorn bulbs induced to produce gum at different times after begin lifted from the soil. The bulbs were either damaged by pricking or not, and were treated with ethylene for 24, 48 or 72 hours as before. It was observed that gum extruded to the outside of the bulb and the internal gum between the bulb scales had a slightly different color and texture. In the later stage of this experiment, the external and internal gum was collected and analyzed separately. The results of this investigation are summarized in Table 3.

In this experiment more gum was produced the longer the duration of ethylene treatment at all time points. In the earliest time point (6 days after harvest), the concentration of 6-tuliposide B increased with longer duration of ethylene treatment, but in the following ones the opposite effect was seen. In the later time-points where internal and external gum was collected separately, it is more difficult to see clear trends. Big differences in tuliposide concentrations were seen, however. In most cases the gum extruded externally had higher concentrations of 6-tuliposide B, although this trend was reversed in some samples.

Table 3. 6-Tuliposide B content in gum and weight of gum of Apeldoorn bulbs induced at different times after harvest. Bulbs were treated with 24, 48 or 72 hours of ethylene gas, and half the bulbs were mechanically damaged. In some samples internal (^{i}) and external (^{e}) gum was collected and analyzed separately. Weight is given as sum of internal and external gum weight.

days after harvest	**pricked**	**6-tuliposide B mg/g gum DW**			**gum production g per 100 g bulbs**		
		24 hr	**48 hr**	**72 hr**	**24 hr**	**48 hr**	**72 hr**
6	no	42.69	68.99	-	0.22	1.22	-
6	yes	26.61	60.90	-	0.19	1.25	-
8	no	239.27	29.69	-	0.60	1.53	-
8	yes	51.16	27.25	-	1.08	1.76	-
13	no	189.22	29.63	50.37	0.75	2.03	2.69
13	yes	119.54	67.03	57.56	1.09	1.97	2.88
27/28	no	119.25	21.11^{i}	46.71^{i}	0.41	1.47	2.67
			259.63^{e}	173.74^{e}			
27/28	yes	45.27	188.06^{i}	15.41^{i}	1.18	2.29	4.90
			26.46^{e}	238.61^{e}			
42/44	no	44.00^{i}	291.46^{i}	182.11^{i}	0.49	0.71	1.97
		196.64^{e}	46.96^{e}	63.10^{e}			
42/44	yes	67.09^{i}	44.35^{i}	53.50^{i}	1.13	3.02	3.32
		171.67^{e}	190.36^{e}	178.67^{e}			

Effect of methyl jasmonate treatments on gum production and tuliposide in gum

The effect of methyl jasmonate application on gum production and 6-tuliposide B concentration in the gum was investigated. The experiment was performed at 37 days after harvest of the bulbs. Methyl jasmonate was applied either as a spray or on filter paper to evaporate into the air in the vat. Although the higher concentration JA-Me spray (2000 ppm) treatment resulted in more gum production compared to the others

(Figure 13a), the methyl jasmonate treatments did not seem to make a big difference to the concentration of 6-tuliposide B in the gum (Figure 13b).

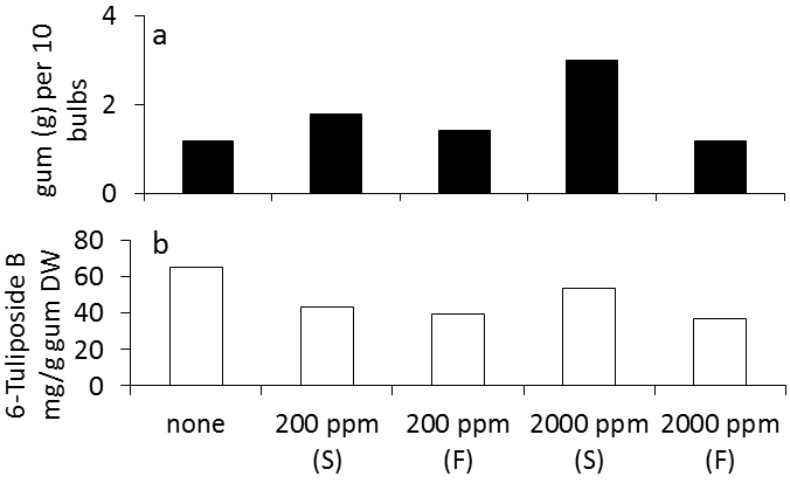

Figure13. Weight of gum (a) and 6-tuliposide B concentration (b) of gum produced by Apeldoorn bulbs induced with 50 ppm ethylene for 24 hours, and different amounts of methyl jasmonate applied either as spray (S) or on filter paper (P). Controls were also treated with 50 ppm ethylene for 24 hours. All samples were treated at 28°C, treatments were done 37 days after harvest.

Discussion

In the process of gummosis gum is made and accumulated in blisters in the tulip bulbs scales. The blisters can burst open so that the gum is extruded, either to the outside of the bulb or into the space between bulb scales. Collection of the gum extruded to the outside is easy, while more effort is required to collect gum between bulb scales and in blisters. In this study the total amount of gum collected per treatment was indicated, but of the total amount sometimes as much as 80% of the mass is from difficult to obtain blisters. Gum produced by cv. Apeldoorn tended to occur more in blisters and between bulb scales, while in cv. Madame Lefeber more gum was extruded to the outside of the bulb. The gum collected in the various treatments varied in texture from softer and gel-like to hard and dry. It was observed that the softer gum, when left exposed to air, would dry out and become hard after a time. All the gum samples were freeze-dried before extraction for NMR analysis to be able to compare 6-tuliposide B content in terms of dry weight. The water content was found to vary between about 7-80% of fresh weight.

As already mentioned, it is known that certain tulip cultivars are more sensitive to ethylene than others. In this study, bulbs of cv. White Dream were the least sensitive to ethylene, and bulbs of cv. Madame Lefeber seemed to be the most sensitive in terms of gum production.

Mechanical damage had previously been observed to increase gum production in certain tulip cultivars (H. Gude personal communication). In this study pricking with a needle was tested as a form of mechanical damage to see if gum production or tuliposide content would be enhanced. In cv. Apeldoorn this mechanical damage had no significant effect on gum production, but the 6-tuliposide B content was lower in gum from damaged samples. No significant effect of needle pricking on gum production or 6-tuliposide B content was seen in gum from cv. Madame Lefeber. When different types of mechanical damage were investigated, none had a positive effect on either gum production or 6-tuliposide B concentration as compared to the control. Cutting the bulbs into four pieces drastically reduced the amount of gum produced.

One effect that can be clearly seen from these experiments is that the duration of exposure to ethylene increases the amount of gum produced. This was the case in all three cultivars investigated. In Apeldoorn the 6-tuliposide B concentration in the gum seemed to follow roughly the opposite trend, decreasing with increased ethylene exposure time. A similar trend seemed to occur in Madame Lefeber samples as well, although this cultivar was not as extensively investigated as cv. Apeldoorn.

In tulip stems, applying JA-Me together with ethylene results in increased gum production (Skrzypek et al., 2005). This enhancement was not seen in our experiment on tulip bulbs. It is possible that the signaling pathways interact differently in the bulbs as compared to the stems. JA-Me applied to the bulbs may be less able to penetrate the cells or to have an effect in this organ.

The absolute values of gum weight and 6-tuliposide B concentration in identical treatments of different experiments were not always the same. For example 6-tuliposide B concentrations in Apeldoorn gum treated for 24 hours with ethylene ranged from about 60-239 mg/g DW in experiments conducted at different times. This is probably related to when the experiments were performed in relation to time of harvest. This has been reported to affect the amount of gum produced in tulips (Kamerbeek et al., 1971). From the results presented here it seems that this also affects the concentration of 6-tuliposide B in the gum.

Induction of gummosis in healthy tulip bulbs was investigated as a potential method of obtaining 6-tuliposide B and related compounds for industrial use. 6-Tuliposide B was

found to be present in all tulip gum samples tested. The production of gum depends on various factors, such as sensitivity of the cultivar to ethylene, duration of exposure to ethylene and time of gummosis induction relative to harvest. With the optimal conditions, it has been shown that gum can be produced from tulip bulbs in this way at amounts more than 6 g gum per 100 g bulbs. As was shown in this study, however, the 6-tuliposide B concentration varies in the gum. It was observed that sometimes the 6-tuliposide B concentration decreased with increased gum production. This suggests that there is a dilution of the small molecule as more gum is produced. It may therefore not be optimal to have long ethylene exposure times to produce large amounts of gum, if the 6-tuliposide B concentration in it will be very low. Depending on the cultivar, gum is mostly accumulated inside blisters and between bulb scales, or otherwise extruded to the outside. For ease of collecting large amounts of gum, cultivars that mostly extrude gum to the outside of the bulb would be better suited for a bigger operation. For truly large-scale operations, a more automatic way of obtaining the gum or the tuliposide from the gum may have to be developed.

This was an initial study to investigate the effects of various induction conditions on the gum production and tuliposide content of the gum. This method has potential for the production of pure 6-tuliposide B from tulips. Production costs would likely be high due to high labor costs, so it would be best suited for the production of the compound for use as a high-value fine chemical. This as opposed to its production as a starting material to make tulipalins for production of bioplastic copolymers, for example, where large amounts of the monomer is needed at low cost. Further studies on structural aspects and properties of the gum itself may reveal uses for the gum as is: a polysaccharide gum containing a small bioactive metabolite. For such use it would be necessary to produce gum with standardized tuliposide content. With careful optimization and control of all conditions involved in producing the gum, this should be possible.

Other bulbs species are also known to produce gum in response to pathogens and/or ethylene, such as species of *Iris* (Liliaceae) and *Hyacinthus* (Hyacinthaceae) (Kamerbeek and De Munk, 1976). Grape hyacinth (*Muscari armeniacum*, Hyacinthaceae) bulbs can be stimulated to produce gum as well (Miyamoto et al., 2010). Gum produced by these and other bulbs may contain interesting small molecules, as these plant families are known to contain many diverse secondary metabolites. The gum may be worth investigating as novel sources of bioactive molecules in relatively pure states.

General conclusions and perspectives

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Ornamental crops are produced in The Netherlands on a very large scale, and cut flowers and bulbs represent some of the country's major export products. Many of the ornamental bulb crops belong to plant families known to contain interesting bioactive chemical compounds. This means that in addition to the ornamental industry, these crops could potentially be cultivated for other industrial purposes. As a major part of this thesis the conversion of an ornamental crop, *Narcissus pseudonarcissus* cv. Carlton to a medicinal crop for the extraction of galanthamine was studied. Following a description of the cultivation of this ornamental crop in The Netherlands, as well as the quality expectations from the pharmaceutical industry, key points critical to the production of high quality raw material for galanthamine extraction were identified.

In Chapter 3 an overview is given of the general aspects that are important to consider in a Good Agricultural Practice (GAP) cultivation scheme. The most important part is the documentation of every step carried out in the production of the plant material, to ensure complete traceability. An example of a document that could be used in such a cultivation scheme for *Narcissus* bulbs is provided in Appendix A. Some key points in the cultivation process were identified that could affect the quality of the bulbs. Experiments were conducted to investigate these key points so that recommendations can be made for a cultivation scheme that would be suitable for GAP.

The first critical step to be considered was the pesticide treatment of the bulbs before planting. The results of a field study showed that the galanthamine levels of bulbs treated with certain fungicides before planting were significantly different from a control treatment. Compared to the control treatment, the pre-planting fungicide treatment most closely representing that as typically done in ornamental cultivation had the highest galanthamine concentration (although not significantly higher than the control). The metabolomics analysis showed that fungicides with different modes of action can cause different alterations in the plant metabolism, still detectable in the bulbs at the end of the growing season. Pesticide application in the field was the next point to consider in the *Narcissus* production chain. Here effects were again seen in the galanthamine levels and metabolite profiles as compared to the control treatment.

Generally lower galanthamine levels were seen, and altered sugar metabolism was also observed. However, when in-field fungicide treatment was combined with an application of mixed fungicides before planting, no lowering of galanthamine was seen. These results showed that while individual fungicide applications could lower the galanthamine levels, the fungicide mixtures typically applied in the production of *N. pseudonarcissus* cv. Carlton does not have a negative effect on the galanthamine in the bulbs. In a screening study of pesticides and herbicides applied before planting and in the field, some residues were detected in the plant material. If fungicides and other protective agents (herbicides, insecticides) are applied during cultivation, this should be documented so that targeted analysis of residues can be conducted. Another step investigated for its effect on galanthamine in the bulbs was the application of fertilizers in the field. The standard nitrogen and potassium fertilizer application as done in ornamental cultivation was found to be optimal for production of galanthamine in the bulbs. Applying more nitrogen or potassium fertilizer did not result in further increases of the alkaloid. Using a metabolomics approach it was seen that various primary metabolite pathways were altered upon increased fertilizer treatment.

Fungal infection, in particular with *Fusarium oxysporum* is a major problem in the cultivation of *Narcissus* in the Netherlands. While it may result in lower yield of bulbs, the possibility exists that the fungi may produce mycotoxins in the bulbs. Bulbs infected with this fungal pathogen were screened for mycotoxin content, and the presence of one, beauvericin was confirmed. This compound is toxic to human cells and thus only healthy bulbs should be used for galanthamine extraction. Since it is not always obvious that bulbs are infected with *F. oxysporum*, targeted screening for beauvericin may be a necessary quality control step as part of a GAP scheme.

Time of harvest may influence the galanthamine levels in the bulbs. Previous pot studies and field studies done in the UK showed that galanthamine in the bulbs were at a maximum around the time of flowering. In a field study monitoring galanthamine over the course of a growing season galanthamine level was found to reach a maximum before flowering. For sustainable production of the bulbs for galanthamine extraction, harvesting at the end of the growing season as for ornamentals is a better option, as this will ensure a good yield and bulb planting stock for the next season. In addition to galanthamine, the changes in the next two most abundant alkaloids, haemanthamine and narciclasine in the leaves, bulbs and roots were also investigated. While not as abundant as galanthamine, the bulbs represent a potential source of these two interesting bioactive compounds. Their extraction from the bulbs in addition to galanthamine could add further value to the bulb as raw material for industrial use.

In the final part of this thesis the discovery of a bioactive small molecule, 6-tuliposide B, in the gum produced by tulip bulbs is described. This was the result of investigations into various ornamental bulb crop materials for compounds for potential industrial use. The process of gummosis is a physiological response to fungal infection, which can also spread to otherwise healthy bulbs in storage due to the release of ethylene. This response is undesirable as it results in lower bulbs yields. The idea was to investigate the small molecule profile of this unwanted "waste" material for interesting components. The presence of tuliposide in the gum at high purity as well as the fact that the gum can be induced in healthy bulbs made this an interesting potential production method of this compound. Studies were carried out to investigate the occurrence of 6-tuliposide B and related compounds in gums of various tulip cultivars. The process of gum induction was also studied in experiments testing various factors that influence gum production and tuliposide content. It was shown that with some careful optimization it would be possible to produce tuliposide-containing gum from the bulbs of certain tulip cultivars for industrial use. From the amounts of gum that it is currently possible to obtain it seems that this would be most suitable for extraction of the small molecule for use as a fine chemical, as opposed to something needed in larger amounts (e.g. biopolymer building block).

The main tool used to monitor the galanthamine and general metabolite profiles in the *Narcissus* bulbs as well as the tuliposides in the tulip gum was ^{1}H NMR. This method was useful as it allowed the quantitative analysis of galanthamine, while simultaneously providing qualitative and quantitative information on metabolites in other compound classes. In the tulip gum it was very useful, as quantification was possible without a reference standard of the compound of interest. It was also possible to obtain structural information about related compounds in the gum. In the *Narcissus* studies, the limitations of the method were mainly related to the relatively small number of metabolites detectable in the crude bulb extracts, and the extensive signal overlap which made signal identification challenging. Two-dimensional NMR experiments were able to aid further resolution of signals and identification of compounds to some extent. For the purposes of the studies conducted, where the target compound galanthamine could be accurately quantified, the method was suitable. For deeper insights into the physiological processes and mechanisms behind the observed results further targeted metabolite analyses would be needed. The combination of ^{1}H NMR metabolomics data with that of other approaches would allow for better understanding of the observed effects. For example, gene expression studies or targeted biosynthetic pathway analysis could help explain why certain changes were seen in galanthamine and other metabolites in response to certain fungicide applications. In the way it was used here ^{1}H NMR analysis is a good analytical method for quantifying target compounds in

optimized plant extracts. As an approach to do metabolomics, as it was used here, it is a good first step for identifying a pathway or group of compounds to investigate for a given experiment. It is therefore a good hypothesis generating tool, but for answering deeper physiological questions additional information from complementary techniques is needed.

Existing crops being produced on large scale, as in the case of the ornamental bulb crops in the Netherlands, represent a good starting point for the search of novel compounds or products for industrial use. The fact that the plant material is already available on a large scale is a major advantage. Also cultivated plants already have well-established cultivation practices, which saves time in comparison to wild plants which have to be brought into cultivation for the first time. The Dutch bulb crops have a further advantage, namely the existence of thousands of cultivars. This large genetic diversity is the result of centuries of hybridizations, and could potentially mean a large chemical diversity to be explored.

Appendix A

Example of record for cultivated *Narcissus* bulbs for pharmaceutical use

Planting material		
Scientific name		
Cultivar		
Source/supplier		
Date of purchase		
Quality of bulbs		
Comments		
Storage, treatment before planting		
Temperature		
Humidity		
HWT	Date	
	Storage temp. before HWT	
	Water temp.	
	Duration treatment	
	Chemicals in water bath	
Pre-planting field		
Site history/crop rotation		
Pests/diseases on previous crops		
Soil properties	Composition (% clay/silt/sand)	
	Soil pH	
	Fertility	Good/poor
	Moisture retention	Good/poor
Irrigation	Yes/no	
	Irrigation type	
	Water source	
	Water quality	Good/poor
	Salt content	High/low
Adjacent crops		
Soil cultivation	Yes/no	
	Date	
	Description	
Soil microflora		
Nutritional status of soil	N	
	P	
	K	
	Mg	
Fertilizer added	Type	
	Quantity	
	Date	
Planting		
Date of planting		
Planting depth		

Planting density		
Arrangement		
In-field operations		
Cover crops	Yes/no	Type
		Density
		Date planted
Crop cover	Yes/no	Type
		Density
		Date applied
Weed control	Herbicide applied	
	Date	
	Amount	
Pesticide	Pesticide applied	
	Date	
	Amount	
	Authorized person	
Irrigation	Yes/no	
	Date	
	Amount	
Unusual circumstances in field		
Bulb lifting		
Date		
Weather conditions		
Days left in field		
Weather conditions		
Date transfer to storage		
Bulbs handling for sale/replanting		
Bulb drying	Temperature	
	Humidity	
Cleaning	Yes/no	
HWT	Date	
	Storage temp. before HWT	
	Water temp.	
	Duration treatment	
	Chemicals in water bath	
Storage	Temperature	
	Humidity	
	Date start of storage	
Quality of bulbs		
Average galanthamine concentration		
Presence of diseases		
Chemical residues		
Mycotoxins		

Summary

In this thesis, the conversion of *Narcissus pseudonarcissus* cv. Carlton from an ornamental crop to a medicinal crop as a source of the alkaloid galanthamine was investigated. The aim of the project was to use this crop as a model system for developing a production chain from planting to raw material for an industrial product. From this project relevant information was obtained that can be applied to other novel industrial crops. A further aim was to investigate other ornamental bulb crops cultivated on large scale as sources of novel natural products.

Plants produce a huge variety of chemical compounds, with diverse structures and properties. Many of these are plant secondary metabolites that are used as pharmaceuticals, flavors and fragrances, colorants, dyes, cosmetics and biocides. Sometimes plants are cultivated as sources of such industrial products, but mostly they are still collected in the wild. The need for renewable sources of industrial products as well as the need to protect plant biodiversity creates an opportunity for farmers to produce medicinal and aromatic crops (**Chapter 1**).

Cultivation of plants as raw material for the pharmaceutical industry (or other industrial uses) differs from cultivation for ornamental purposes. In the latter plants are cultivated mainly for the flowers, and the most important aspects are flower quality and yield. For medicinal crops, the content of the target metabolite is the most important quality aspect. In addition, factors related to the safety of the plant material are also important, such as the presence of toxic agrochemical residues and mycotoxins. Most important is the incorporation of all cultivation practices and procedures into a Good Agricultural Practice scheme, which allow complete traceability of the production chain (**Chapter 3**). On the basis of how *Narcissus* is typically cultivated in The Netherlands for ornamental use, various experiments were performed to see how these practices affect the bulb quality in terms of galanthamine and other aspects.

NMR-based metabolite analysis and profiling was chosen as the major tool to analyze the *Narcissus pseudonarcissus* cv. Carlton plant material. ^{1}H NMR analysis has the advantages of being relatively quick to perform, and requiring minimal sample preparation. For crude plant extracts, structural and quantitative information can be obtained for various types of metabolites simultaneously. Limitations to sensitivity restrict the number of metabolites that can be detected in a crude extract, while signal overlap can hamper signal assignment and compound identification. However, the use of two-dimensional NMR experiments enables deconvolution of overlapped signals and confirmation of signal assignments. Multivariate data analysis methods can be applied

to ^{1}H NMR spectra to compare all detectable metabolite signals between many plant samples at the same time. This is useful for simplifying the NMR data and extracting information about the variance in a given dataset (**Chapter 2**).

A quantitative ^{1}H NMR method was developed to allow the accurate determination of galanthamine in the bulbs of *N. pseudonarcissus* cv. Carlton (**Chapter 4**). The method was validated and was found to be comparable to an existing validated HPLC method for galanthamine quantitation. Bulbs from different cultivation areas were used in this experiment, and the overall ^{1}H NMR spectra obtained were compared using a metabolomics approach. Based on the results, the overall metabolite profiles of bulbs grown in different regions were similar, but were discriminated based on galanthamine levels.

A field experiment was conducted to determine how fertilizer applications affect galanthamine levels in *N. pseudonarcissus* cv. Carlton bulbs (**Chapter 5**). The standard fertilization applications of nitrogen and potassium caused a significant increase in galanthamine as compared to a control. Multivariate data analysis of the ^{1}H NMR data revealed that applying double the standard level of nitrogen fertilizer resulted in production of more amino acids and citric acid cycle intermediates, but not more galanthamine. The results indicated that standard levels of fertilizer currently applied in The Netherlands are sufficient for optimal galanthamine accumulation in the bulbs. The major diseases of cultivated *N. pseudonarcissus* cv. Carlton are caused by fungal pathogens, which usually make application of fungicides necessary. The effects of fungicides on galanthamine and other metabolites of *N. pseudonarcissus* cv. Carlton bulbs were investigated in a field experiment (**Chapter 6**). In some fungicide treatments, significantly lower galanthamine levels were seen. Multivariate data analysis revealed further changes in bulb metabolite patterns caused by fungicides. Bulbs treated before planting generally had higher levels of alkaloids, while foliar field applications caused lower alkaloid levels and altered carbohydrate metabolism. Within these groups, specific metabolites were altered by certain individual fungicide treatments. The fungicides used in *Narcissus* cultivation can cause a change in the metabolome still detectable in the bulbs after harvest. However, the standard cultivation practices in terms of fungicide treatment were found suitable for the production of *N. pseudonarcissus* cv. Carlton as raw material for galanthamine extraction.

The high input of fungicides, herbicides and insecticides in the ornamental cultivation of *N. pseudonarcissus* cv. Carlton means that residues of these agrochemicals may remain in or on the plant material. In the strictly regulated pharmaceutical industry every potential impurity in the process of production needs to be characterized and

proven to be safe. It is therefore important to know whether there are pesticide residues in the raw starting material. In a field study pesticides and herbicides were applied to *N. pseudonarcissus* cv. Carlton plants at various stages of the production process (**Chapter 7**). Since ^{1}H NMR was not sensitive enough for these analyses, LC-MS and GC-MS based methods were used to determine the levels of residues where possible. Of the pesticides and herbicides tested, traces of several were detected in the bulb material. Three fungicides were found at levels high enough for quantitation. These results showed that residues of certain agrochemicals may remain in or on the plant material, underlining the importance of keeping track of all pesticide and herbicide applications in a GAP scheme. Some of the fungi that infect *Narcissus* bulbs in the Netherlands belong to genera that are known to produce harmful mycotoxins. The presence of mycotoxins may be an issue when cultivating *Narcissus* as pharmaceutical raw material and may be an important part of a GAP scheme. *Narcissus pseudonarcissus* bulb samples heavily infected with *Fusarium oxysporum*, the most common fungal pathogen, were analyzed for the presence of mycotoxins (**Chapter 7**). One mycotoxin, beauvericin, was present in the infected bulb samples. This compound is toxic to human cells, therefore care should be taken to use only healthy bulbs for the extraction of galanthamine, and screening for this mycotoxin should also be part of a GAP scheme.

After galanthamine the next two most abundant alkaloids in the bulbs of *N. pseudonarcissus* cv. Carlton are haemanthamine and narciclasine. In a field study the levels of galanthamine, haemanthamine and narciclasine were monitored in the bulbs, leaves and roots of *N. pseudonarcissus* plants throughout the growing season (**Chapter 8**). The leaves had high levels of the alkaloids before flowering. The bulbs had relatively lower levels of alkaloids per dry weight, but due to larger biomass would yield higher total amounts of the compounds when extracted after the usual harvest time. *N. pseudonarcissus* cv. Carlton is already being cultivated for the extraction of galanthamine, but also represents a source of haemanthamine and narciclasine. In addition to the alkaloid quantitation, multivariate data analysis of the ^{1}H NMR spectra also revealed changes in metabolites in the different plant organs over time. The leaf chlorogenic acids and flavonoids changed significantly over the course of the growing season. In the roots there was a large increase in phenolic compounds at the end of the growing season. From the time course of the metabolite profiles it appears that the alkaloids are synthesized in the bulbs and leaves of the plant, but are transported from other organs to the roots.

In a search for novel industrial products from ornamental crops produced in the Netherlands, tulip bulb gum was analyzed for small metabolite content. Tulip gum is the result of a physiological process called gummosis that occurs in certain tulip cultivars in

response to fungal infection. The gum has not been characterized in terms of small metabolites before, and it was found to contain 6-tuliposide B in a relatively pure form. This compound and related tuliposides and tulipalins have various reported bioactivities. Together with its relatively pure state in the gum, this makes the gum a potential source of this compound for industrial use. Experiments were performed to assess how the production of gum containing 6-tuliposide B can be optimized. A quantitative ^{1}H NMR method was developed to analyze tuliposide in the gum. The results showed that with careful optimization of parameters (such as tulip cultivar, duration of exposure of bulbs to ethylene, time of gummosis induction after harvest and mechanical damage) the gum can be produced in large enough amounts to be a source of 6-tuliposide B (**Chapter 9**).

The results presented in this thesis contribute to the knowledge needed to convert an ornamental crop to a medicinal or other industrial crop. The production of *Narcissus* bulbs for extraction of galanthamine is a good example of how crops already cultivated on a large scale can be used for new purposes. The discovery of a small bioactive metabolite in tulip gum further illustrates the potential of existing ornamental bulb crops to be developed as sources of novel industrial products.

Samenvatting

In dit proefschrift wordt het onderzoek beschreven hoe de narcis *Narcissus pseudonarcissus* cv. Carlton de overstap kan maken van een sierplant naar een medicinale plant, als bron van het alkaloid galanthamine. Het doel van dit project was het gebruik van deze plant als een model voor het opzetten van een productielijn van het planten aan de ene kant tot het in handen krijgen van het ruwe product aan de andere kant. Uit dit project werd waardevolle informatie verkregen die gebruikt kan worden bij de ontwikkeling van andere nieuwe industrieel te telen gewassen. Een tweede doel van het project was het onderzoeken van andere op grotere schaal geteelde bolgewassen als bron van nieuwe natuurstoffen.

Planten produceren een scala aan chemische verbindingen, met veelal sterk gevarieerde structuren en eigenschappen. Veel van deze stoffen zijn zgn. secundaire metabolieten die worden gebruikt als medicijnen, smaak- of geurstoffen, kleurstoffen, verven, cosmetica en biocides. In sommige gevallen worden voor deze doeleinden de planten geteeld, maar de meeste worden nog steeds in het wild verzameld. De behoefte aan een constante bron voor deze industriële producten, maar ook de noodzaak om plantenbiodiversiteit te beschermen geven kwekers de mogelijkheid om zulke medicinale soorten te kweken (**Hoofdstuk 1**).

De kweek van planten voor medicinale (of andere industriële) doeleinden verschilt van de gebruikelijke sierteelt. Laatstgenoemde richt zich vrijwel enkel op de bloemen, wat de bloemkwaliteit en –opbrengst de belangrijkste factoren maakt. Bij medicinale planten is het gehalte in de plant van de doelstof de belangrijkste factor die de kwaliteit bepaalt. Ook zijn in dit geval veiligheidsaspecten veel belangrijker, zoals restanten van gebruikte gewasbeschermende middelen en toxines geproduceerd door plantenziekteverwekkers. Belangrijk is mede daarom, om de gehele kweekprocedure vast te leggen in een GAP-protocol (Good Agricultural Practice), zodat de teelt van stap tot stap kan worden gevolgd (**Hoofdstuk 3**). Met de in Nederland gangbare standaardteeltmethode voor narcissen als uitgangspunt werd een aantal experimenten gedaan om te zien hoe variatie in teeltomstandigheden tot uiting kwam in verschillen in het gehalte aan galanthamine en andere eigenschappen.

Als belangrijkste middel werd analyse met behulp van NMR gebruikt om plantenmateriaal van *Narcissus pseudonarcissus* cv. Carlton te karakteriseren. ^{1}H-NMR heeft als grote voordeel dat weinig monstervoorbewerking hoeft plaats te vinden en analyses snel uitgevoerd kunnen worden. Zowel kwalitatieve als kwantitatieve metingen in ruwe plantenextracten kunnen zo tegelijkertijd uitgevoerd worden. Het aantal stoffen dat op deze manier kan worden aangetoond is echter beperkt door de detectiegrenzen en

het probleem van overlappende signalen van de diverse inhoudsstoffen. Deze problemen kunnen echter deels worden overkomen door het gebruik van tweedimensionele NMR, zodat de signalen toch juist kunnen worden toegewezen, resulterend in een juiste identificatie. Met multivariate data analysemethoden kunnen ^{1}H NMR-gegevens worden gebruikt om alle signalen van de verschillende detecteerbare metabolieten in een groot aantal plantenpreparaten tegelijkertijd te meten. Dit is bijzonder nuttig om de complexe NMR-data te vertalen in informatie over de variatie in een gegeven dataset (**Hoofdstuk 2**).

Een kwantitatieve ^{1}H-NMR methode werd ontwikkeld om een nauwkeurige bepaling van gelanthamine in de bollen van *N. pseudonarcissus* cv. Carlton mogelijk te maken (**Hoofdstuk 4**). Deze methode werd gevalideerd, en bleek gelijkwaardig aan een reeds bestaande gevalideerde HPLC-methode voor de bepaling van galanthamine. Bollen van verschillende bronnen werden aldus geanalyseerd middels een metabolome analyse met behulp van de opgenomen ^{1}H-NMR-spectra. De resultaten toonden gelijkwaardige metabolietprofielen van de bollen uit verschillende regio's, al waren er duidelijke verschillen in de galanthaminegehaltes.

In een veldexperiment werd bestudeerd hoe verschillende vormen van bemesting van invloed waren op het galanthaminegehalte van bollen van *N. pseudonarcissus* cv. Carlton (**Hoofdstuk 5**). Standaardbemesting met stikstof en kalium bleek een significant hoger gehalte te geven in vergelijking met een controle. Verdubbeling van de stikstofbemesting resulteerde echter volgens een multivariate data-analyse van ^{1}H-NMR-gegevens in de productie van meer aminozuren en intermediairen uit de citroenzuurcyclus, maar niet in meer galanthamine. De standaardhoeveelheden die in Nederland gebruikt worden voor bemesting zijn blijkbaar voldoende voor een optimale galanthamineproductie in de bollen.

Schimmels zijn de belangrijkste ziekteverwekkers waarmee rekening moet worden gehouden bij de teelt van *N. pseudonarcissus* cv. Carlton. Dit maakt het gebruik van fungicides noodzakelijk. De effecten van deze middelen op de productie van galanthamine en andere metabolieten in *N. pseudonarcissus* cv. Carlton werden onderzocht in een veldexperiment (**Hoofdstuk 6**). Belangrijk lagere hoeveelheden galanthamine werden gevonden bij het gebruik van sommige (maar niet alle) fungicides. Door middel van multivariate data-analyse kon worden aangetoond dat er meerdere veranderingen optraden in het metabole patroon van de bollen door het gebruik van fungicides. Hogere alkaloidgehaltes werden gevonden indien de bollen waren behandeld vóór het planten, terwijl behandeling van de uitgekomen planten juist resulteerde in lagere gehaltes, alsmede een veranderd koolhydraatmetabolisme. Ook

werden er verschillen gevonden in de patronen van metabolieten bij gebruik van verschillende fungicides. Zulke verschillen in profielen konden ook na oogst van de bollen nog worden teruggevonden. De gebruikelijke methode van fungicidebehandeling bleek echter goed genoeg als standaard voor de galanthamineproductie door *N. pseudonarcissus* cv. Carlton.

Het gebruik in de teelt van *N. pseudonarcissus* cv. Carlton van fungicides, herbicides en insecticides betekent dat er restanten van deze chemicaliën achterblijven in of op het plantenmateriaal. De strikte regelgeving in de farmaceutische industrie vereist dat elke mogelijke verontreiniging in het productieproces moet worden geïdentificeerd en aangetoond moet worden dat deze geen gevaar betekent. Het is om die reden belangrijk om te weten of er pesticideresten in het ruwe plantenmateriaal aanwezig zijn. In **Hoofdstuk 7** wordt beschreven hoe in een veldstudie met *N. pseudonarcissus* cv. Carlton pesticides en herbicides worden gebruikt in verschillende stadia van de teelt. Daar 1H-NMR niet gevoelig genoeg was om de restanten van de middelen op te sporen, werden hiervoor LC-MS en GC-MS als analysemethode gebruikt. Van een aantal geteste pesticides en herbicides werden sporen teruggevonden in het bolmateriaal. Drie van de geteste fungicides konden worden aangetoond in kwantificeerbare hoeveelheden. Deze resultaten toonden aan dat er restanten agrochemicaliën in plantenmateriaal achter kunnen blijven, hetgeen het belang onderstreept om in een GAP-protocol nauwkeurig bij te houden welke middelen er worden gebruikt. Sommige in Nederland bekende schimmels die narcissen kunnen besmetten zijn berucht vanwege de productie van schadelijke mycotoxines. In een GAP-protocol moet derhalve rekening gehouden worden gehouden met de mogelijke aanwezigheid van zulke toxines als het materiaal als farmaceutische grondstof aangeleverd zal gaan worden. De aanwezigheid van mycotoxines werd nader onderzocht in bollen van *Narcissus pseudonarcissus* die sterk geïnfecteerd waren met *Fusarium oxysporum*, de algemeenste pathogene plantenschimmel (**Hoofdstuk 7**). Één mycotoxine, beauvericine, kon worden aangetoond in de geïnfecteerde bollen. Deze stof is toxisch voor humane cellijnen, en de afwezigheid ervan moet derhalve altijd gecontroleerd worden in een GAP-protocol. Ook moet worden afgezien van het gebruik van bollen waarin infectie wordt vermoed.

Na galanthamine zijn haemanthamine en narciclasine de algemeenste alkaloiden in bollen van *N. pseudonarcissus* cv. Carlton. In een veldstudie werden de gehaltes van al deze drie alkaloiden in bollen, bladeren en wortels van *N. pseudonarcissus* gemonitord over het gehele teeltseizoen (**Hoofdstuk 8**). De bladeren vertoonden een hoog alkaloidgehalte vóór het bloeien. De bollen hadden een relatief lage concentratie aan alkaloiden op basis van drooggewicht, maar omdat de bollen veruit de grootste biomassa van de plant vertegenwoordigen levert dit deel toch het meest aan alkaloiden

op bij extractie na de gebruikelijke oogsttijd. *N. pseudonarcissus* cv. Carlton wordt nu reeds geteeld voor de extractie van galanthamine, maar is ook een goede bron voor haemanthamine en narciclasine. Multivariate data-analyse van ^{1}H-NMR-spectra toonde aan dat de metabolieten in verschillende plantenonderdelen varieerden in de tijd. Zo waren er aanmerkelijke verschillen in chlorogeenzuren en flavonoiden in de bladeren gedurende het teeltseizoen. In de wortels was een sterke toename van fenolische verbindingen te zien aan het eind van het seizoen. Uit de metabolietprofielen over de tijd blijkt dat de alkaloiden worden gesynthetiseerd in de bollen en bladeren, en getransporteerd naar de wortels.

Tulpenbollenextract werd geanalyseerd op kleine metabolieten in een inventarisatie naar mogelijke nieuw te ontwikkelen producten uit in Nederland gekweekte sierplanten. Het extract is een soort gom die door sommige tulpencultivars in een proces genaamd gummosis wordt geproduceerd als respons op schimmelinfecties. Deze tulpengom is niet eerder gekarakteriseerd voor wat betreft de kleine metabolieten die erin voorkomen. Het bleek dat hij 6-tuliposide-B bevatte van een vrij hoge zuiverheid. Deze stof, alsmede verwante tuliposides en tulipalines vertonen verschillende vormen van bioactiviteit, hetgeen tulpengom interessant maakt als bron voor industriële ontwikkeling van de inhoudsstof(fen). Experimenten werden uitgevoerd om de productie van de tuliposide bevattende gom te optimaliseren. Zo werd een ^{1}H-NMR-methode ontwikkeld voor de kwantitatieve bepaling van tuliposide in de gom. Uit de resultaten kon worden afgeleid dat door nauwkeurige optimalisatie van verschillende parameters (zoals tulpcultivar, lengte van blootstelling van de bollen aan ethyleen, de duur van inductie van de gummosis na de oogst en beschadiging) deze gom in voldoende grote hoeveelheden kan worden gewonnen voor de productie van 6-tuliposide B (**Hoofdstuk 9**).

De resultaten beschreven in dit proefschrift dragen bij aan de benodigde kennis voor het gebruik van sierteeltplanten als bron van medicinale of anderszins bruikbare inhoudsstoffen. De teelt van narcissenbollen voor de productie van galanthamine is een goed voorbeeld van het gebruik van reeds op grote schaal geteelde sierteeltgewassen voor nieuwe doeleinden. De ontdekking van een bioactieve metaboliet in tulpengom bevestigt het potentieel dat bolgewassen hebben als bron van nieuw te ontwikkelen industriële producten.

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Curriculum vitae

Andrea Lubbe was born in Belville, South Africa on 7 June 1983. After completing a Bachelor of Science (BSc.) degree in Molecular and Cellular Biology at Stellenbosch University in 2004, she continued with an Honours degree in Botany at Stellenbosch University. During her undergraduate and Honours degrees at Stellenbosch she developed an interest in medicinal plants.

After graduating from Stellenbosch University in 2005 (BSc. Hons.) she was awarded a Huygens Scholarship from the Dutch organization Nuffic to follow the Master of Science (MSc.) program in Natural Products and Biodiversity at Leiden University in the Netherlands. Her MSc research involved the bioactivity-guided fractionation of medicinal plant *Sceletium tortuosum*, and phytochemical studies on the plant's alkaloids.

She graduated from the MSc. program in 2008, and stayed in Leiden to continue working towards a PhD degree under the supervision of Prof. Robert Verpoorte. Her PhD research was part of two projects, the first funded by the Flowers and Food Foundation and the second funded by the province of Zuid-Holland. Her work was focused on the cultivation of *Narcissus pseudonarcissus* (daffodils) as raw material for the extraction of the alkaloid galanthamine as a drug against Alzheimer's disease. The broader topic of her doctoral research was the conversion of Dutch ornamental bulb crops to medicinal or other industrial crops. The results of this research are presented in this thesis. During her PhD studies (December 2010-May 2011), she spent six months working at Qiagen GmbH in Hilden, Germany as part of Marie Curie Industry-Academic exchange Metabolomics Standardization Project. Here she obtained valuable experience working in a large life sciences company.

With the sound knowledge of phytochemical techniques and metabolomics approaches obtained in Leiden, her aim is to continue studying medicinal plants, and to gain experience in other "omics" techniques. On completion of her doctoral studies, she will continue with post-doctoral research on plant secondary metabolites important to human health at Washington State University in the United States.

List of Publications

Andrea Lubbe, Henk Gude, Robert Verpoorte, Young Hae Choi (2013) Seasonal accumulation of major alkaloids in organs of pharmaceutical crop *Narcissus* Carlton. *Phytochemistry*, in press.

Andrea Lubbe, Robert Verpoorte, Young Hae Choi (2012) Effects of fungicides on galanthamine and metabolite profiles in *Narcissus* bulbs. *Plant Physiology and Biochemistry* 58, 116-123.

Andrea Lubbe, Isabell Seibert, Thomas Klimkait, Frank van der Kooy (2012) Ethnopharmacology in overdrive: The remarkable anti-HIV activity of *Artemisia annua*. *Journal of Ethnopharmacology* 141 (3), 854-859.

Andrea Lubbe, Kashif Ali, Robert Verpoorte, Young Hae Choi (2012) NMR-based metabolomics analysis, in Metabolomics in Practice: Successful Strategies to Generate and Analyze Metabolic Data, edited by Michael Lämmerhofer and Wolfram Weckwerth (Wiley-VCH, Weinheim) (In press).

Andrea Lubbe, Young Hae Choi, Peter Vreeburg, Robert Verpoorte (2011) Effect of Fertilizers on Galanthamine and Metabolite Profiles in *Narcissus* Bulbs by 1H NMR. *Journal of Agricultural and Food Chemistry* 59 (7), 3155-3161.

Andrea Lubbe, Robert Verpoorte (2011) Cultivation of Medicinal and Aromatic Plants for specialty industrial materials. *Industrial Crops and Products* 34, 785-801.

Arno Hazekamp, Justin T. Fischedick, Mónica Llano Díez, **Andrea Lubbe**, Renee L. Ruhaak (2010) Chemistry of Cannabis, in *Comprehensive Natural Products II*, Volume 3, Second Edition, edited by Lew Mander and Hung-Wen Lui (Oxford: Elsevier Ltd, 2010). Chapter 3.24, 1033-1084.

Andrea Lubbe, Barbora Pomahacova, Young Hae Choi, Robert Verpoorte (2010) Analysis of Metabolic Variation and Galanthamine Content in *Narcissus* Bulbs by 1H NMR. *Phytochemical Analysis* 21 (1) 66-72.

Patents:

Robert Verpoorte, **Andrea Lubbe**, Hendrikus Gude (2012), WO/2012/057617, Method for the production of glycosides from bulbs and use of the glycosides thus produced. Application filed October 2011.