Analytical and phytochemical studies on valerian and valerian based preparations

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Print: Offsetdrukkerij Ridderprint B.V., Ridderkerk

RIJKSUNIVERSITEIT GRONINGEN

ANALYTICAL AND PHYTOCHEMICAL STUDIES ON VALERIAN AND VALERIAN BASED PREPARATIONS

PROEFSCHRIFT

ter verkrijging van het doctoraat in de
Wiskunde en Natuurwetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, Dr. F. van der Woude,
in het openbaar te verdedigen op
vrijdag 21 november 1997
des namiddags te 4.15 uur

door

Reinder Bos

geboren op 5 april 1944
te Appingedam

Promotores: Prof. Dr. H. V. Wikström
Prof. Dr. J. J. C. Scheffer

Referent: Dr. H. J. Woerdenbag

Stellingen

Behorende bij het proefschrift

Analytical and phytochemical studies on valerian and valerian based preparations

1. Het gebruik van 1,8-dihydroxyanthrachinon (danthron) als interne standaard bij de kwantitatieve analyse van valereenzuur, dat is voorgesteld voor de monografie van '*Valerianae radix*' in de Europese Farmacopee, doet gezien de aard van de verbinding afbreuk aan de nauwkeurigheid van de bepaling.

2. Het apparaat voor de bepaling van het gehalte aan vluchtige olie dat beschreven is in de Nederlandse Farmacopee 6^{e} uitgave, 2^{e} druk, verdient vanwege zijn robuustheid en vanwege de hoge mate van reproduceerbaarheid die ermee bereikt kan worden, de voorkeur boven het apparaat beschreven in de Europese Farmacopee 3^{e} editie.

3. Het identificeren van vluchtige sesquiterpenen was en is moeilijk, zoals moge blijken uit de op massaspectrometrie gebaseerde identificatie van elemol - dat later valerianol bleek te zijn - door Hazelhoff *et al.* en de op verschillende methoden gebaseerde, door Bos *et al.* voorgestelde structuur van een verbinding met een retentie-index van 1555 uit de olie van *Valeriana wallichii*, die wellicht identiek is aan spirojatamol, zoals wordt beweerd door Marschall en Weyerstahl.
   B. Hazelhoff *et al.* (1979) *Pharm. Weekbl.* **114**, 443-449; R. Bos *et al.* (1997) *Flavour Fragr. J.* **12**, 123-131 (dit proefschrift, hoofdstuk 5); H. Marschall en P. Weyerstahl, persoonlijke mededeling.

4. De kwaliteit van de wetenschappelijke bewijsvoering voor de werkzaamheid van fytotherapeutica op basis van valeriaan vormt een schril contrast met de omvang van het gebruik van deze preparaten.

5. Bij het gebruik van fytotherapeutica is de veiligheid van de preparaten in eerste instantie belangrijker dan een goed gedocumenteerd bewijs voor hun klinische effectiviteit.

6. Met behulp van *in-vitro* cytotoxiciteitsassays kan de aanwezigheid van mogelijk toxische stoffen in fytotherapeutica op eenvoudige wijze snel worden aangetoond, zelfs wanneer zij in lage concentraties voorkomen.

7. Ondanks de groeiende belangstelling - nationaal en internationaal - voor fytotherapie krijgt deze vorm van therapie zeer weinig aandacht binnen de farmaceutische opleiding in Nederland.

8. Ten gevolge van de geringe instroom van jong analytisch geschoold personeel in het universitair onderwijs en onderzoek, zal er bij de pensionering van de huidige garde een schat aan praktische ervaring verloren gaan, omdat deze niet of nauwelijks kan worden overgedragen.

9. De verbouwing van het voormalige Tandheelkunde-gebouw ten behoeve van het Universitair Centrum voor Farmacie in Groningen heeft zeker niet voor iedere werkgroep de beoogde voordelen opgeleverd.

10. Voor een goede en krachtige economische ontwikkeling van de gezamenlijke Eemshavens is een gemeentelijke herindeling een eerste vereiste.

11. Ook in de dubbelrol van OBP-er (OBP = ondersteunend beheerspersoneel en oudere, bijzondere promovendus) kan een promotie-onderzoek tot een goed einde worden gebracht.

Groningen, 21 november 1997 Rein Bos

Aan mijn ouders

Voor Jeanne

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Financial support by the Van Leersumfonds for the investigations described in the chapters five and six is gratefully acknowledged.

Printing of this thesis was sponsored by the Verenigde Nederlandse Kruidencoöperatie ua, Elburg, The Netherlands.

Cover page: Wilde Valeriaene, from *Bloemlezing uit het Cruydt-boeck*, R. Dodoens, p. 96.

ISBN 90-9010948-X

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Preface

Valerian species belong to the oldest medicinally used plants. Extracts from the roots and rhizomes have been used in the treatment of several ailments already in antiquity. In the middle of the eighteenth century, the drug was used for the first time for its supposed sedative properties. *Valeriana officinalis* is cultivated as a medicinal plant on a commercial scale in the northern part of Europe, and The Netherlands plays an important role here.

Chemical analyses were carried out for the first time in the nineteenth century, and were especially directed to the essential oil. In the search for the active substances of valerian, many compounds have been isolated and identified during the last 120 years. Despite the fact that many of these components have been pharmacologically tested, it is yet unclear which group(s) of components is (are) responsible for the sedative effect of the plant. Irrespective of this, several clinical studies have pointed to a sedative activity. On the basis of this, it has to be adopted that the activity rests on the interaction of several individual components (Hölzl, 1996).

The Department of Pharmaceutical Biology (formerly: Pharmacognosy) of the University Centre for Pharmacy in Groningen has a 'long tradition' with regard to *V. officinalis*. Over the past 25 years, many investigations took place. It started with the quantitative examination of the essential oil for selection purposes, and was later extended with the quantitative HPLC analysis of valerenic acid and its derivatives thereof. As a result, several papers have been published. Laufer *et al.* (1970) investigated the biologically active components in several *Valeriana* and *Centranthus* species. Isovaltral, an artifact of isovaltrate, was isolated and identified in 1979 by Denee *et al.* Studies on phytochemical and pharmacological aspects of valerian compounds, with special reference to valepotriates, resulted in a PhD thesis by Hazelhoff in 1984. The last two decades, the essential oil, valerenal, and valerenic acid and its derivatives have become more important, not only from a pharmacological point of view, but also for the quality control and standardization of phytomedicines. The results of investigations concerning the occurrence of valeranone and cryptofauronol in the essential oil of *V. officinalis* collected in the northern part of The Netherlands and the pharmacological screening of valerenal, valerenic acid and other components of the essential oil were published by Hendriks *et al.* between 1980 and 1985.

Studies of the variation in the essential oil content and composition in individual plants obtained after breeding experiments with a *V. officinalis* strain can be regarded as the beginning of more extensive experiments resulting in this PhD thesis.

The present study aims to characterize valerian species and valerian preparations with respect to their contents of valerenic acid and its derivatives, valepotriates, and essential oil components. Special attention is paid to the analysis of plant material and of commercially available phytomedicines with respect to valerenic acid and its derivatives, valepotriates and baldrinals. Also the composition of the essential oils of several other valerian species has been studied. In addition, attention is paid to breeding experiments and improvement of the quality of the crude plant material, in order to get insight into the seasonal variation of the characteristic groups of secondary metabolites of the plant, and for the determination of the optimum harvest time of the roots and rhizomes which is important from a commercial point of view. Finally, the cytotoxic potential of characteristic valerian constituents and valerian tinctures has been evaluated, in view of the safety of valerian and valerian preparations.

Chapter 1

VALERIAN AND VALERIAN BASED PHYTOMEDICINES: BOTANY, CHEMISTRY, ANALYSIS, PHARMACOLOGY AND USE[1]

Introduction

The subterranean parts of three valerian species, namely *Valeriana officinalis* L. *s.l.*, *V. wallichii* DC. and *V. edulis* Nutt. ex Torr. & Gray, are used as mild sedatives, but show large differences with regard to their constituents. Consequently, phytomedicines prepared from these species are characterized by different chemical compositions (Hänsel, 1990; Bos *et al.*, 1994; 1996).

It is still not fully clear which constituents are responsible for the sedative action, but valepotriates as well as valerenic acid and derivatives thereof are generally considered to contribute to it (Houghton, 1988; Morazzoni and Bombardelli, 1995; Bos *et al.*, 1996). In addition, the essential oil may play a role in the biological activity of valerian (Hazelhoff *et al.*, 1979a; Bos *et al.*, 1994; 1996). Quality assurance and quality control of the crude drug and its preparations should therefore be based on these major groups of secondary metabolites.

In this chapter, botanical, chemical and analytical aspects with regard to the medicinally used valerian species are discussed as well as their use, pharmacology and toxicology.

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[1]This chapter is based on:
Bos R, Woerdenbag HJ, Hendriks H, Malingré ThM (1992) Der indische oder pakistanische Baldrian, *Valeriana wallichii* DC. (= *Valeriana jatamansi* Jones). *Z. Phytother.* **13**, 26-34.
Bos R, Woerdenbag HJ, Zwaving JH (1994) Valeriaan en valeriaanpreparaten. *Pharm. Weekbl.* **129**, 37-43.
Bos R, Woerdenbag HJ, De Smet PAGM, Scheffer JJC (1997) *Valeriana* species. In *Adverse Effects of Herbal Drugs*, Vol. 3 (De Smet PAGM, Keller K, Hänsel R, Chandler RF, eds), Springer-Verlag, Berlin, Heidelberg, pp. 165-180.
Woerdenbag HJ, Bos R, Scheffer JJC (1997) Quality assurance of the crude drug and its preparations. In *Valerian, the Genus Valeriana* (Houghton PJ, ed.), Harwood Academic Publishers, Amsterdam, pp. 101-128.

Botany

The genus *Valeriana* belongs to the Valerianaceae and contains about 250 species. The majority of representatives of this genus is distributed over the temperate regions of the Old World, but they also occur in Central and South America. The three most important species that play a role in herbal medicine are *V. officinalis* L. *s.l.*, *V. wallichii* DC. (synonym *V. jatamansi* Jones) and *V. edulis* Nutt. ex Torr. & Gray ssp. *procera* (H.B.K.) F.G. Meyer (synonym *V. mexicana* DC.; Mexican valerian) (Hänsel, 1990; Hölzl, 1996). *V. edulis* Nutt. ex Torr. & Gray can be divided geographically in three different subspecies: *V. edulis* ssp. *ciliata* (Torr. & Gray) F.G. Meyer; *V. edulis* ssp. *edulis*; and *V. edulis* ssp. *procera* (H.B.K.) F.G. Meyer (Reichling *et al.*, 1994).

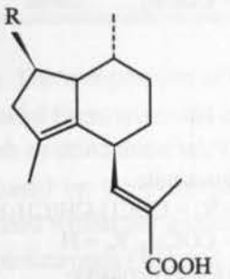
V. officinalis L. *s.l.* (valerian) is listed in the European Pharmacopoeia. Valerian roots consist of the subterranean organs of *V. officinalis* L. *s.l.* including the rhizomes, roots and stolons (Anonymous, 1997). In Europe this species is cultivated on a large scale for the preparation of pharmaceuticals, prepared from the subterranean parts (Bos *et al.*, 1996). *V. officinalis* is a collective term; hence the addition *s.l.* (*sensu lato*). Several subspecies are distinguished within the species, e.g. *V. collina* Wallr. (synonym *V. officinalis* L. ssp. *collina* (Wallr.) Nyman), *V. exaltata* Mikan ex Pohl (synonyms *V. officinalis* L. *s. str.*, *V. altissima* Hornemann, *V. officinalis* L. var. *altissima* Koch, *V. muticeps* Wallr., *V. officinalis* L. var. *major* Koch sensu Vocke & Angelrodt, *V. palustris* Kreyer, *V. provisa* Kreyer), *V. pratensis* Dierbach ex Walther (synonym *V. officinalis* L. var. *pratensis* Dierb.), *V. procurrens* Wallr. (synonym *V. repens* Host) and *V. sambucifolia* Mikan fil. ex Pohl (synonym *V. excelsa* Poir.) (Titz and Titz., 1981; Titz *et* al., 1982; Reichling *et al.*, 1994). The subspecies differ morphologically and cytologically, as well as in area of distribution (Steinegger and Hänsel, 1992). Other subspecies mentioned in the literature that belong also to the collective species are *V. salina* Pleijl, *V. officinalis* L., *V. alternifolia* Ledeb., and *V. sylvestris* Grosch (Weberling, 1970; Hegnauer, 1973; Penso, 1983; Frohne and Jensen, 1992; Steinegger and Hänsel, 1992; Bruneton 1995).

V. wallichii DC., the Indian or Pakistani valerian, is listed in the Indian Pharmacopoeia. It is collected in the Himalayas (Evans, 1989).

V. edulis Nutt. ex Torr. & Gray ssp. *procera* (H.B.K.) F.G. Meyer originates from Central America and is described in the Mexican Pharmacopoeia (Lorenz, 1989; Hölzl, 1996).

Chemistry

V. officinalis contains valerenic acid (**1**) and its derivatives hydroxyvalerenic acid (**2**) and acetoxyvalerenic acid (**3**), as well as valtrate (**4**), isovaltrate (**5**) and acevaltrate (**6**). Small amounts of didrovaltrate (**7**) and isovaleroxyhydroxy-didrovaltrate (IVHD; **8**) may also be present (Thies, 1968a; Titz *et al.*, 1982; 1983; Bos *et al.*, 1997a). (The valepotriate structures are according to El-Nagger and Beal, 1980)



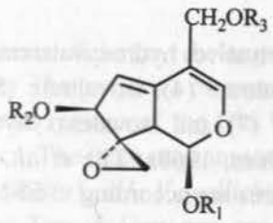
1 Valerenic acid
R = H

2 Hydroxyvalerenic acid
R = OH

3 Acetoxyvalerenic acid
R = $OCOCH_3$

V. wallichii and *V. edulis* lack valerenic acid and derivatives, but contain valepotriates in higher contents than *V. officinalis*. In *V. wallichii*, valtrate, isovaltrate and didrovaltrate are found (Wienschierz, 1978; Hölzl, 1996; Bos *et al.*, 1992). In *V. edulis*, valtrate, isovaltrate, acevaltrate, didrovaltrate and isovaleroxyhydroxydidrovaltrate are present (Thies, 1968a; Lorenz, 1989).

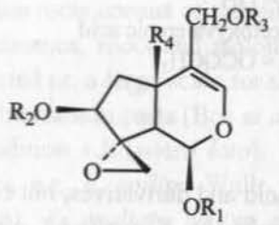
Essential oil can be isolated from *V. officinalis* (Hazelhoff *et al.*, 1979a; Hendriks and Bos, 1984; Bos *et al.*, 1986a; 1986b; 1997c) and *V. wallichii* (Bos *et al.*, 1992; 1997b; Hölzl, 1996). In *V. edulis* only trace amounts of volatiles are present (Hendriks and Bos, 1984; Steinegger and Hänsel, 1992; Bos *et al.*, 1997f). In Table 1, a survey is given of the contents of these groups of compounds in the three valerian species just mentioned, as reported in the literature. All three species are used for the production of solid, oral dosage forms, while from *V. officinalis* also tinctures and a tea are made (Bos *et al.*, 1996).



4 Valtrate
$R_1 = R_2 = COCH_2CH(CH_3)_2$
$R_3 = COCH_3$

5 Isovaltrate
$R_1 = R_3 = COCH_2CH(CH_3)_2$
$R_2 = COCH_3$

6 Acevaltrate
$R_1 = COCH_2C(CH_3)_2OCOCH_3$
$R_2 = COCH_2CH(CH_3)_2$
$R_3 = COCH_3$



7 Didrovaltrate
$R_1 = R_3 = COCH_2CH(CH_3)_2$
$R_2 = COCH_3$, $R_4 = H$

8 Isovaleroxyhydroxy-didrovaltrate (IVHD)
$R_1 = COCH(OCOCH_2CH(CH_3)_2)$-$CH(CH_3)_2$
$R_2 = COCH_2CH(CH_3)_2$
$R_3 = COCH_3$, $R_4 = OH$

Another relevant group is formed by the baldrinals, yellow-coloured decomposition products of the valepotriates. Baldrinal (**9**) originates from valtrate and acevaltrate; homobaldrinal (**10**) from isovaltrate (Steinegger and Hänsel, 1992; Bos *et al.*, 1997a). As cytotoxic and mutagenic effects have been described for the baldrinals (Bounthanh *et al.*, 1981; Braun *et al.*, 1986; Von der Hude *et al.*, 1986; Dieckmann, 1988), their absence in the crude drug and in preparations has to be proved in order to avoid possible hazardous effects (Bos *et al.*, 1996).

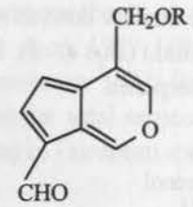
The subterranean parts of the three medicinally used *Valeriana* species show large differences with regard to their constituents. Dried roots and rhizomes of *V. officinalis* contain 0.5-2.0% (v/w) of essential oil, which is mainly composed of mono- and sesquiterpenoids. More than 150 compounds have been found in the oil so far. They include acyclic, monocyclic and bicyclic hydrocarbons, as well as oxygen-containing derivatives, such as alcohols, aldehydes, ketones, phenols, oxides and esters (Hendriks and Bos, 1984; Bos *et*

Table 1. Contents - based on dry weight - of the major groups of compounds in three valerian species used in herbal medicine (Hölzl, 1996; Bos *et al.*, 1997a)

	Valerenic acid and derivatives (%)		Valepotriates (%)		Essential oil (%, v/w)	
Valeriana	Hölzl	Bos *et al.*	Hölzl	Bos *et al.*	Hölzl	Bos *et al.*
officinalis	0.08-0.3	0.05-0.9	0.5-2.0	0.8-1.7	0.3-0.7	0.5-2.0
wallichii	absent	absent	3.0-6.0	1.8-3.5	0.1-3.0	0.1-0.9
edulis	absent	absent	4.0-7.0	8.0-12.0	< 0.10	< 0.10

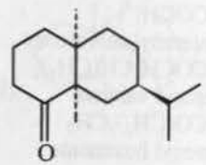
al., 1996). The composition of the oil is highly influenced by the origin of the plant material (genotype, soil and climate), and by the method of isolating the oil (from fresh or dried material, via extraction or distillation).

Based on the principal component of the oil, four chemotypes can be distinguished within the species *V. officinalis*, namely valeranone (**11**), valerianol (**12**), cryptofauronol (**13**) and valerenal (**14**) types (Bos *et al.*, 1986a). Initially, the valerianol type was called elemol type (Hazelhoff *et al.*, 1979a; Hendriks and Bruins, 1980; Hendriks *et al.*, 1977).

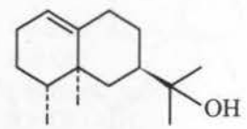


9 Baldrinal
$R = COCH_3$

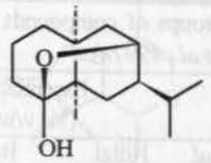
10 Homobaldrinal
$R = COCH_2CH(CH_3)_2$



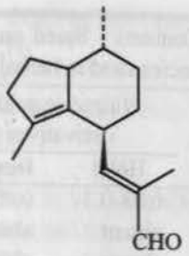
11 Valeranone



12 Valeraniol

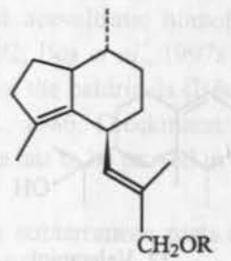


13 Cryptofauronol

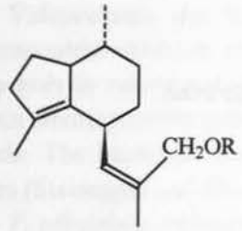


14 Valerenal

V. officinalis contains characteristic cyclopentane sesquiterpenes. In the essential oil valerenal, valerenol (**15**) and its acetate (**16**), isovalerate (**17**), valerate (**18**) and hexanoate esters (**19**), and small amounts of valerenic acid and its methyl ester have been found (Bos *et al.*, 1986b; 1997c; 1997e). Important non-volatile cyclopentane sesquiterpenes are valerenic acid and its derivatives acetoxyvalerenic acid and hydroxyvalerenic acid. The first two compounds are specific for *V. officinalis* (Hänsel and Schulz, 1982), while the third one is probably formed when the root material is stored improperly, e.g. under too humid conditions. In that case hydroxyvalerenic acid is produced from acetoxyvalerenic acid. The content of valerenic acid and its derivatives ranges from 0.05% (in wild plants) to 0.9% (in cultivated strains) (Bos *et al.*, 1997d). *V. wallichii* and *V. edulis* lack these cyclopentane sesquiterpenes.



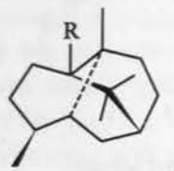
15 Valerenol
R = H
16a *E*-Valerenyl acetate
R = $COCH_3$
17a *E*-Valerenyl isovalerate
R = $COCH_2CH(CH_3)_2$
18 Valerenyl valerate
R = $CO(CH_2)_3CH_3$
19 Valerenyl hexanoate
R = COC_5H_{11}



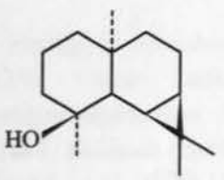
16b *Z*-Valerenyl acetate
R = $COCH_3$
17b *Z*-Valerenyl isovalerate
R = $COCH_2CH(CH_3)_2$

The essential oil of *V. wallichii* (0.1-0.9%, v/w) contains mainly the sesquiterpene alcohols patchouli alcohol (**20**) and maaliol (**21**) (Bos *et al.*, 1992; 1997b). *V. edulis* contains only small amounts of essential oil, e.g. patchouli alcohol (Bos *et al.*, 1997f). The volatile compounds that are obtained from the subterranean parts of this plant by distillation mainly include valeric, isovaleric and hydroxyvaleric acids, as well as several other decomposition products formed upon heating of valepotriates (Hendriks and Bos, 1984; Steinegger and Hänsel, 1992; Hölzl, 1996).

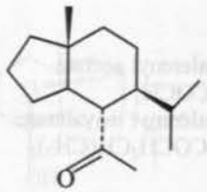
V. officinalis and *V. wallichii* have several constituents in common with Japanese valerian species. They include valeranone (jatamansone), faurinone (**22**), kessane (**23**), α-kessyl acetate (**24a**), cryptofauronol, fauronyl acetate (**25**), maaliol and patchouli alcohol (Stoll *et al.*, 1957a; Govindachari *et al.*, 1958; Krepinsky *et al.*, 1959; Hikino *et al.*, 1963; 1968; Narayanan *et al.*, 1964; Rücker and Kretzschmar, 1972; Bos *et al.*, 1983; Woerdenbag *et al.*, 1997). It has been found that the total terpene content in Japanese valerian species is 2-25 times higher than in European species (Suzuki *et al.*, 1993).



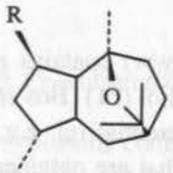
20 Patchouli alcohol
R = OH
20a Patchoulyl acetate
R = $OCOCH_3$



21 Maaliol



22 Faurinone



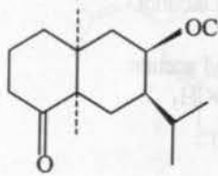
23 Kessane
R = H

24 α-Kessyl alcohol
R = OH

24a α-Kessyl acetate
R = $OCOCH_3$

24b α-Kessyl isovalerate
R = $OCOCH_2CH(CH_3)_2$

In the 1960s, Thies and coworkers (Thies, 1966; Thies and Funke, 1966) isolated a novel group of natural products from subterranean parts of *V. wallichii*, and called these compounds valepotriates. Valepotriates are triesters of polyalcohols with an iridoid structure which possess an epoxy group (valeriana-epoxy-triesters). Differences are found in the number of hydroxyl groups, the type of ester groups, an the degree of saturation. As a result of dehydration or esterification of the various alcohol functions, species-dependent mixtures of valepotriates are yielded. Based on their chemical structure, valepotriates are divided into two main groups, namely the diene type (including valtrate, isovaltrate and acevaltrate) and the monoene type (including didrovaltrate and isovaleroxyhydroxydidrovaltrate) (Bos *et al.*, 1992; Steinegger and Hänsel, 1992).



25 Fauronyl acetate

Valepotriates are instable compounds: they are thermolabile and decompose under acidic or alkaline conditions, as well as in alcoholic solutions. After hydrolysis valeric and isovaleric acids are found among other compounds. The main decomposition products of the valepotriates are the yellow-coloured baldrinals. The baldrinals are chemically reactive and may subsequently form polymers (Steinegger and Hänsel, 1992; Bos *et al.*, 1996).

V. officinalis contains 0.8-1.7% of a mixture of valepotriates, consisting mainly of valtrate and isovaltrate in a ratio of 1:1-1:4 (Bos *et al.*, 1997d). *V. wallichii* contains 3-6% of valepotriates (Hölzl, 1996). Next to valtrate and isovaltrate, didrovaltrate is also present in this species. Two chemotypes are distinguished for *V. wallichii*: the monoene and diene types (Wienschierz, 1978; Hölzl, 1996). *V. edulis* is the richest in valepotriates (8-12%). Valtrate, isovaltrate, acevaltrate, didrovaltrate and IVHD are present here (Thies, 1968a; Lorenz, 1989). Valerosidate, an iridoid glycoside, is found in *V. officinalis* (up to 1.5%) and in *V. wallichii* (up to 5%) (Steinegger and Hänsel, 1992). Valepotriates are not only present in *Valeriana* species but also in *Centranthus* species. In addition, they do not occur exclusively in subterranean parts of the plants, but also in the leaves of *Valeriana* and *Centranthus* species (Funke and Friedrich, 1974; 1975; Hölzl and Jurcic, 1975; Petricic, 1979; Dieckmann, 1988; Houghton, 1988).

In contrast to the valepotriates, valerenic acid and its derivatives are chemically stable. In the future, they may play an important role in the standardization of valerian preparations prepared from *V. officinalis* (De Smet and Vulto, 1988; Hänsel, 1992; Bauer *et al.*, 1994; Bos *et al.*, 1996). Using the guidelines of the German Pharmacopoeia (9th edition), Schimmer and Röder (1992) investigated valerian roots and valerian tincture. Valerenic acids were detected by thin-layer chromatography (TLC) in 19 (out of 23) commercial plant drugs derived from *V. officinalis*. In two plant products containing extracts from *V. wallichii* DC. and *V. edulis* Nutt. ssp. *procera*, these compounds could not be detected. Valerenic acid and its derivatives were also detected in several self-prepared aqueous extracts and in commercial tinctures (Schimmer and Röder, 1992).

Finally, in the subterranean parts of *V. officinalis* a number of alkaloids (0.05-0.1%) occur; actinidine, 8-methoxyactinidine (valerianine) and naphthyridylmethylketone have been found as well as several other, yet unidentified, alkaloids (Borkowski and Lutomski, 1961; Torssell and Wahlberg, 1967; Franck *et al.*, 1970; Gross *et al.*, 1971; Johnson and Waller, 1971; Cionga

et al., 1976; Buckova *et al.*, 1977; Janot *et al.*, 1979). Furthermore, isoferulic acid, γ-aminobutyric acid, free fatty acids and short-chain carboxylic acids have been isolated (Stoll and Seebeck, 1957; Bos *et al.*, 1997d). In the leaves of *V. officinalis*, the presence of four flavonoids has also been demonstrated (Greger and Ernet, 1971). From the subterranean parts of *V. wallichii*, two isomers of lanarin isovalerate and 4-methoxy-8-pentyl-1-naphtholic acid have been isolated (Thies, 1968b; Chari *et al.*, 1977; Pande and Shukla, 1993).

The differences in secondary metabolites between the three medicinally used *Valeriana* species imply that the pharmaceuticals prepared from the respective crude drugs also largely differ with regard to their chemical composition. In spite of that, legal demands do not exist on this point. However, several manufacturers have standardized their products, either on valepotriates or on valerenic acid and its derivatives. Valerian preparations with a standardized valepotriate content are mostly prepared from *V. wallichii* and *V. edulis*, because these species are relatively rich in valepotriates (Wichtl, 1989). Those standardized on valerenic acid and derivatives are made from *V. officinalis*.

Another important factor is the dosage form. When a herbal tea was prepared by extraction of the subterranean parts of *V. officinalis* with hot water, up to 60% of the valepotriates remained in the root material and only 0.1% could be recovered from the tea (Hänsel and Schulz, 1985). In another study using subterranean parts of *V. officinalis*, no valepotriates could be detected in the tea, whereas valerenic acid and derivatives were present (Bos *et al.*, 1996). This leads to the conclusion that teas from subterranean parts of *V. officinalis* will be practically devoid of baldrinals (Hänsel and Schulz, 1985; Dieckmann, 1988; Bos *et al.*, 1996). For other valerian species (that are not commonly used in the form of a tea) no data are available on this point. However, as *V. wallichii* and *V. edulis* contain considerably larger amounts of valepotriates than *V. officinalis*, it cannot be assumed that a tea prepared from *V. wallichii* or *V. edulis* will also be devoid of baldrinals.

Valerian film-coated tablets and capsules contained small amounts (≤ 1 mg) of baldrinal (Hänsel and Schulz, 1985; Dieckmann, 1988; Bos *et al.*, 1996). Tinctures prepared from *V. officinalis* were devoid of valepotriates already within three weeks after preparation, due to the low stability of these compounds in ethanolic solutions (Petricic, 1979; Hänsel and Schulz, 1985; Bos *et al.*, 1996). In view of this rapid degradation, it is not surprising that no baldrinals could be recovered from commercially available tincture samples (Dieckmann, 1988; Bos

et al., 1996). It is assumed that the baldrinals react further to form condensation products with other constituents from the tincture.

Pharmacopoeial aspects

Crude drug and tinctures

Pharmacopoeial aspects for the crude drug include a definition, macroscopical and microscopical descriptions of the crude drug, identity and purity tests, and an assay for a quantitative determination.

Valerianae radix is listed in the current European Pharmacopoeia, 3rd edn (Anonymous, 1997). Hence, this monograph is also part of, among others, the German Pharmacopoeia, the French Pharmacopoeia, the British Pharmacopoeia, and the Dutch Pharmacopoeia. *Valeriana radix* is defined as the subterranean organs of *V. officinalis* L. *s.l.*, including the rhizomes, roots and stolons, carefully dried at a temperature below 40°C. It contains not less than 0.5% (v/w) of essential oil.

Valerian was also included in the second edition of the Indian Pharmacopoeia (Anonymous, 1966a) and defined as the dried rhizomes, stolons and roots of *V. wallichii* DC. This monograph is not included in the current third edition. *V. edulis* has been included in the Mexican Pharmacopoeia. No monograph on valerian is found in the United States Pharmacopoeia, USP 23/National Formulary, NF 18.

The European Pharmacopoeia 3rd edn (Anonymous, 1997) furthermore contains a general monograph on tinctures, that is also applicable to *Tinctura Valerianae*. However, very limited information is included as yet. According to this pharmacopoeia, tinctures are prepared by maceration, percolation or other suitable, validated methods, using ethanol of a suitable concentration. Tinctures are obtained using 1 part of drug and 10 parts of extraction solvent, or 1 part of drug and 5 parts of extraction solvent. Limits are given for methanol and 2-propanol: not more than 0.05% (v/v) of each of these alcohols is allowed. It is furthermore stated that a tincture should comply with limits prescribed for relative density, ethanol content and drug residue, however, without giving the limits. At this moment they are different for each tincture.

According to the Dutch Pharmacopoeia 6th edn, 2nd printing (Anonymous, 1966b), valerian tincture is prepared by maceration from 1 part of valerian root and 5 parts of ethanol [70%, v/v]. The tincture has a brown colour,

and its relative density is 0.897-0.907 ($d^{20}/_{20}$). The drug residue is at least 2.5% (w/v).

According to the German Pharmacopoeia, 'Deutsches Arzneibuch', DAB 10 (Anonymous, 1993b), valerian tincture is prepared by percolation of 1 part of valerian root with 5 parts of ethanol [70%, v/v]. The ethanol content is 63.5-69.0% (v/v). The drug residue is at least 3.0% (w/v).

Recently, we studied the influence of different ethanol:water ratios on the composition of valerian extracts made from *V. officinalis*. At an ethanol concentration of 30% (v/v), valerenic acid and its derivatives started to be extracted from the subterranean parts. Above 50% (v/v) of ethanol the amounts of these sesquiterpenoids were more or less constant. Valepotriates were extracted only with ethanol concentrations above 70% (v/v). This means that if ethanol [70%, v/v] is used to prepare a valerian tincture, the extraction of the valepotriates is far from complete (Bos *et al.* 1996).

Macroscopical and microscopical descriptions of the crude drug

- Valeriana officinalis

In the European Pharmacopoeia 3rd edn (Anonymous, 1997) the morphology and the microscopical characteristics of the crude drug are described.

Valerian root has a characteristic and penetrating odour, resembling that of valeric acid and camphor. This is particularly true for poorly dried or old material. The taste is somewhat sweet at first, then spicy and slightly bitter. Properly dried material has the odour of the fresh essential oil, without a note of valeric acid.

It is difficult to see the differences under the microscope between the pulverized roots of the three valerian species of interest. Thin-layer chromatography, to determine the presence of valerenic acid, is the best method to prove the identity of *V. officinalis* (Wichtl, 1989).

- Valeriana wallichii

The Indian Pharmacopoeia 2nd edn (Anonymous, 1966a) describes the macroscopical characteristics of the crude drug as follows. Indian valerian consists of dull yellowish-brown rhizomes, 4-8 cm long and 4-10 mm thick, and a very variable amount of roots up to 7 cm long and 1-2 mm thick. The rhizomes are unbranched and somewhat flattened dorsiventrally. The upper surface bears leaf scars and the lower surface roots or root scars. The rhizome breaks with a

short fracture, and the horny interior shows a small dark bark, a well-marked cambium, about 12 to 15 light-coloured xylem bundles and a dark pith and medullary rays. Stolons connect the rhizomes, are stout, 1-5 mm long and 2-4 mm thick, yellowish-grey in colour, longitudinally wrinkled usually with nodes and internodes and bearing adventitious roots. Occasionally thin stolons, 1-2 mm thick, are found. Roots are yellowish-brown, 3-5 cm long and 1 mm thick. The odour is strong and reminiscent of isovaleric acid. The taste is bitter and somewhat camphoraceous (Evans, 1989). A description of the plant is given by Bos *et al.* (1992).

The Indian Pharmacopoeia 2nd edn (Anonymous, 1966a) describes the microscopical characteristics of the crude drug as follows. The rhizome-cork consists of four to 14 layers of lignified suberised cells, that occasionally contain oil globules. The cortex is parenchymatous and contains numerous starch grains, oil globules and a yellowish-brown substance. The outer two or three layers of cortex are collenchymatous and occasional root traces appear as paler strands. The endodermis is one layered. The pericycle is parenchymatous and within it 12 to 18 collateral vascular bundles, separated by dark medullary rays, are present. The pith is large, parenchymatous, lacunar and contains starch grains. Starch occurs as single or occasional compound grains of two components, individual grains being 7-30, mostly 10-25 μm, in diameter. Calcium oxalate is absent.

Stolon-cork consists of two to five layers; the cortex up to 25 layers, parenchymatous, followed by about 20 collateral vascular bundles, which in young stolons are separated by cellulosic parenchymatous medullary rays, which in older stolons become lignified. Pith is wide and lacunar. Root traces are absent. Roots have a small central parenchymatous pith surrounded by tetrarch to polyarch xylem and a wide parenchymatous bark.

- Valeriana edulis

No macroscopical and microscopical descriptions are known. A botanical description of the plant is given by Reichling *et al.* (1994).

Chemical identity and purity tests

Using the identity reaction for *V. officinalis* according to the European Pharmacopoeia 3rd edn (Anonymous, 1997) the presence of valepotriates is shown. Valepotriates are lipophilic compounds that are extracted from the drug by methylene chloride. In the acetic acid-hydrochloric acid mixture used, blue-

coloured cyclopenta-[c]-pyrylium salts (pseudoazulenes) are formed from valepotriates with a conjugated diene structure (valtrate, isovaltrate, acevaltrate) (Böhme and Hartke, 1979).

This identity reaction, however, is not specific for *V. officinalis*. The roots of other *Valeriana* species, which also contain valepotriates of the diene type, will give a positive reaction as well (Hartke and Mutschler, 1987). A conclusion about the identity, however, will be possible after other tests have been carried out. The identity of *V. officinalis* can be based on the purity test of the European Pharmacopoeia 3rd edn (Anonymous, 1997), using TLC (see below). In that test the presence of valerenic acid and derivatives, which are characteristic of *V. officinalis*, is checked. In addition, HPLC methods are available to determine the characteristic valepotriate composition of the crude drug, as well as valerenic acid and its derivatives. These assays, however, are not (yet) included in the European Pharmacopoeia.

- Thin-layer chromatography (TLC)

According to the European Pharmacopoeia 3rd edn (Anonymous, 1997) the purity of *Valerianae radix* is determined by TLC, using silica gel as the coating substance (stationary phase).

The chromatogram obtained with the test solution shows in the middle, at a retention factor (R_f) value between those of the pink zone (Sudan red G) and of the orange zone (aminoazobenzene) in the chromatogram of the reference solution, a deep-violet zone (valerenic acid) and sometimes above this zone a greyish-brown zone (valtrate and isovaltrate). Furthermore, a faint violet zone (acetoxyvalerenic acid) with an R_f value lower than that of the zone due to aminoazobenzene is seen, as well as grey zones situated between the zone due to valerenic acid and the starting point, a number of violet zones of variable intensity in the upper part of the chromatogram, and a mostly very faint violet zone immediately above the starting point.

Because valepotriates as well as valerenic acid and its derivates were not commercially available as reference substances until recently, the location of the spots due to these compounds is related to the location of Sudan red G and aminoazobenzene. Nowadays (iso)valtrate and valerenic acid and its derivatives can be purchased. The purity test is selective for *V. officinalis*, because the presence of valerenic acid and its derivatives is checked (Hartke and Mutschler, 1987). In addition, essential oil components are also separated using this system.

- Extractable matter

The extractable matter of valerian roots, as described in the European Pharmacopoeia 3rd edn (Anonymous, 1997), is not less than 15.0%.

In the European Pharmacopoeia 1st edn (Anonymous, 1975b), this demand was included in the definition of valerian root. However, the determination of the amount of extractable matter is not a substitute for an assay, quantitating valepotriates and/or valerenic acid and its derivatives (Böhme and Hartke, 1978). Moreover, after harvesting the fresh roots are washed. If the washing procedure has taken too long, the roots can leach, resulting in a lower amount of extractable matter (Hartke and Mutschler, 1987).

According to the Indian Pharmacopoeia 2nd edn (Anonymous, 1966a), the alcohol [60%] soluble extractive should not be more than 30% for *V. wallichii*.

- Sulphated ash

Valerianae radix yields not more than 15.0% of sulphated ash, according to the European Pharmacopoeia 3rd edn (Anonymous, 1997).

When organic matter is incinerated as such, the residue found will depend on the temperature used. For instance, alkali chlorides and earth-alkali carbonates are volatile at certain temperatures. In the presence of sulphuric acid non-volatile sulphates are formed. Because pyrosulphates may be formed during the heating procedures, ammonium carbonate is added at the end. The sulphated ash is a measure for the total amount of inorganic matter in the plant material (Böhme and Hartke, 1978).

- Ash insoluble in hydrochloric acid

Valerianae radix yields not more than 7.0% ash insoluble in hydrochloric acid, according to the European Pharmacopoeia 3rd edn (Anonymous, 1997).

Using this procedure, non-volatile, inorganic impurities are determined, such as soil and sand. It is a control of the washing procedure of the crude drug (Hartke and Mutschler, 1987).

Tincture residue

A determination of the residue of *Tinctura Valerianae* is also described in the European Pharmacopoeia 3rd edn (Anonymous, 1997). The result is calculated as a percentage weight over weight (w/w) or weight over volume (w/v).

Results of residue determinations obtained in our laboratory for the three *Valeriana* species discussed in this chapter are: *V. officinalis* 28-31%; *V. wallichii* 21-46%; *V. edulis* 29-30%.

Identity of Tinctura Valerianae

In the German Pharmacopoeia, DAB 10 (Anonymous, 1993b), the identity of valerian tincture is determined by TLC with silica gel as the stationary phase. As a reference solution, fluorescein and Sudan red G are dissolved in methanol.

The chromatogram of the test solution shows - after spraying with anisaldehyde and heating - an intense blue zone at about the R_f of fluorescein due to hydroxyvalerenic acid, and a violet zone at about the R_f of Sudan red G of valerenic acid. In the upper part of the chromatogram several other faint red to violet coloured zones are found.

In a tincture prepared from *Valeriana* species other than *V. officinalis*, the zones of valerenic acid and hydroxyvalerenic acid are absent.

This assay is similar to the TLC analysis described by Hänsel *et al.* (1983), with the difference that these authors used a solution of vanillic acid and of anisaldehyde in methanol as the reference solution. To valerenic acid an R_f value of 0.4-0.5 (just above anisaldehyde) was assigned, and to hydroxyvalerenic acid an R_f value of 0.07-0.12 (just below vanillic acid).

Valerenic acid and acetoxyvalerenic acid, that are characteristic of *V. officinalis*, can be detected immediately in the tincture. However, a purification step is added to the procedure since tinctures that are prepared from Indian or Mexican valerian contain neutral substances that can disturb the detection of the two acids. Therefore, the acids are converted into their corresponding potassium salts. The disturbing neutral compounds can now be removed from the aqueous phase by extraction with methylene chloride. After acidification, the sesquiterpene carboxylic acids are extracted with methylene chloride. This procedure to check the identity of valerian tincture can also be used to prove its purity, because aldulterations are recognized with this procedure. It should be noticed that hydroxyvalerenic acid is not a genuine constituent of *V. officinalis*;

the acid originates from acetoxyvalerenic acid that is hydrolysed in the alkaline aqueous phase by warming.

Purity of the crude drug and of a freshly prepared homoeopathic mother tincture

In the German Homoeopathic Pharmacopoeia, 'Homöopathisches Arzneibuch', HAB 1 (Anonymous, 1975c), a TLC method is described as a purity test for *Valerianae radix* as well as for a freshly prepared mother tincture. The same test was described in the European Pharmacopoeia 1st edn (Anonymous, 1975b).

The TLC analysis for purity can be used to investigate a possible adulteration of *V. officinalis* with the other species; this might be done deliberately in order to enhance the valepotriate content. *V. officinalis* mainly contains valepotriates of the diene type (valtrate, isovaltrate and some acevaltrate), whereas both *V. wallichii* and *V. edulis* contain also considerable amounts of monoene valepotriates (didrovaltrate and IVHD). Valepotriates with a diene structure appear with blue to greenish-grey colours; monoene valepotriates with yellow to greenish-grey colours (Böhme and Hartke, 1979).

V. wallichii and *V. edulis* may contain valepotriate hydrins, yielding violet-blue zones in the chromatogram that are located immediately under the zone of the diene valepotriates mentioned in the previous paragraph (Böhme and Hartke, 1979). Valepotriate hydrins have the same chemical structure as valepotriates, but an acid is added to the epoxy group (Thies, 1968a).

Van Meer *et al.* (1977) pointed to the fact that next to the valepotriates located in the lower half of the chromatogram, components of the essential oil of valerian root are present, which are located in the upper half, i.e. above the zone due to valtrate. The essential oil components can be distinguished from the valepotriates by first spraying the chromatogram with a mixture of glacial acetic acid-hydrochloric acid [25%] 1:1. The essential oil components colour purple to violet, whereas valepotriates yield green-grey (valtrate, acevaltrate), blue-green (IVHD) and blue-grey spots (unidentified valepotriates with lower R_f values). After subsequent spraying of the chromatogram with dinitrophenylhydrazine reagent, two components of the oil (aldehydes and/or ketones; R_f 0.65 and 0.80) are coloured yellow to yellow-orange. After a while, two other components (R_f 0.88 and 0.95) that do not belong to the essential oil colour yellow. These components were not seen before in daylight, nor under UV light or after spraying with acid. After spraying with the acid, under valtrate also purple zones are visible.

Identity of a stored homoeopathic mother tincture of valerian root

The HAB 1 (Anonymous, 1975c) also comprises a TLC identity test for a stored mother tincture, that differs from a freshly prepared mother tincture. Valepotriates are not detectable in a stored tincture. Hence, constituents of the essential oil are analysed. Silica gel is used as the stationary phase, and the stored mother tincture as test solution. As a reference solution, borneol and bornyl acetate are dissolved in methanol.

Qualitative and quantitative determinations

Valepotriates

- Spectrophotometry

The first procedure for a quantitative determination of valepotriates has been described by Mannestätter *et al.* (1968). They isolated 'Halazuchrom B' (= valtrate) from a valerian extract, prepared with diethyl ether, by TLC on silica gel with methylene chloride-methyl ethyl ketone 9:1, or a column chromatographic separation with hexane. Valtrate was measured spectrophotometrically at 254 nm.

Valtrate was also converted into a deep-blue coloured product using hydrochloric acid in methanol ('Halazuchrom-Reaktion') whereafter the absorbance was measured at 610 nm (Mannestätter *et al.*, 1968). The identity reaction for *V. officinalis*, as described in the European Pharmacopoeia 3rd edn (Anonymous, 1997), is derived from this earlier work.

As only diene valepotriates colour blue with hydrochloric acid, didrovaltrate cannot be determined in this way. In addition, didrovaltrate does not have a UV maximum at 254 nm. Wagner *et al.* (1970) described a spectrophotometric method by which all valepotriates can be determined. After a TLC separation of the valepotriates, followed by extraction, the hydroxylamine-iron(III) chloride reaction was carried out, after which the absorbance was measured at 512 nm.

Because of their epoxide structure, valepotriates are able to alkylate the nucleophilic agent 4-(4')-nitrobenzylpyridine (NBP). This reaction is linear time- and dose-dependent and can therefore be used for a specific quantitative assay of valepotriates using a suitable standard (Braun *et al.*, 1983). Of a sample and a reference solution (30 mg valtrate or didrovaltrate in 10 ml methanol), 50 µl were incubated in a water-bath of 40°C for 90 min with 2.95 ml of NBP-reagent (360

mg NBP dissolved in 2.8 ml of methanol, 7.0 ml of Tris-HCl-buffer [0.1 N; pH 7.4] and 11.0 ml of ethylene glycol). Subsequently, to 2.0 ml of the reaction mixture 1.5 ml of a solution of tetrene (tetramethylene pentamine) in acetone (2 + 3) were added. After mixing, the absorbance was measured 30 s later, at 560 nm.

- Titrimetry

The first titrimetric determination of valepotriates has also been described by Mannestätter *et al.* (1968), namely the 'Jodhydrin-Methode'. The epoxy group of the valepotriates was opened with sodium iodide in an ethanolic, acetate-buffered, acetic acid solution and then the corresponding iodine hydrine was formed.

As this reaction proceeds very slowly (24 h), Liptak and Verzár-Petri (1980) published an improved method with respect to time. The titration of valepotriates was performed by opening the epoxide ring with hydrobromic acid; the reaction mixture was rapidly titrated with 0.01 N sodium acetate solution. Just before the end of the titration 5 ml of a crystalviolet solution was added as an indicator. The titration was continued until a blue colour appeared.

- Thin-layer chromatography (TLC)

Several TLC methods for the determination of valepotriates, qualitatively as well as quantitatively, are found in the literature. Stahl and Schild (1969) published the first complete instruction for quickly testing valerian root for its valepotriates.

Powdered valerian root, 0.2 g, was extracted with 5 ml of methylene chloride. After filtration and washing the filter with 2 ml of the same solvent, the solvent was evaporated on a water-bath and the residue subsequently taken up in 0.2 ml of methanol. Of this solution, 10 µl were used for chromatography. As a reference solution 10 mg of vanillin and 10 µl of anisaldehyde were dissolved in 10 ml of methanol. Of the reference solution, 5 µl were used for chromatography.

As stationary phase silica gel with a fluorescence indicator (GF_{254}) was used. The plate was developed twice over a path of 10 cm, using hexane-methyl ethyl ketone 8:2 as the mobile phase, in a saturated chamber. The spots were visualized under UV light (254 nm) and after spraying with benzidine-hydrochloric acid reagent (0.1% benzidine in hydrochloric acid [25%]-glacial acetic acid 1:1), followed by 10 min heating at 110°C (Stahl and Schild, 1971).

Under UV light, the largest zone is found at R_f 0.5-0.6 (equal to anisaldehyde), due to valtrate. After the reaction with benzidine a series of coloured zones are seen. Valtrate becomes green-grey and anisaldehyde yellow. In the lower part of the chromatogram a blue zone is found at R_f 0.2-0.3 (equal to vanillin, yellow). Between the blue zone and the valtrate zone, two smaller and less intensively coloured zones are visible, due to didrovaltrate and acevaltrate.

The applied spray reagent was regarded an improvement if compared with older reagents. Spraying with 6 N hydrochloric acid (Mannestätter *et al.*, 1967) yielded an instable blue colour with valtrate and acevaltrate. Also a mixture of equal volumes of hydrochloric acid [25%] and glacial acetic acid only coloured the diene valepotriates (Thies and Funke, 1966). Antimony trichloride [22%, w/v] in chloroform (Thies and Funke, 1966; Mannestätter *et al.*, 1967) did not give clear colours in daylight.

Benzidine is a carcinogenic agent; as a substitute, Stahl and Schild (1969) proposed a solution of 0.1% 2,4-dinitrophenylhydrazine in hydrochloric acid [25%]-glacial acetic acid 1:1 as a spraying reagent. As a disadvantage, a yellow background was obtained and the fluorescence was less; as an advantage, the intensity of the colours due to valtrate, acevaltrate (blue) and didrovaltrate (faint orange) was stronger.

Other mobile phases reported to give a good separation of valepotriates include benzene-ethyl acetate 9:1, light petrol-ethyl acetate 8:2, diethyl ether-hexane 5:5, light petrol-acetone 8:2 (Stahl and Schild, 1969).

Laufer *et al.* (1970) developed an improved TLC analysis for valepotriates, based on the method of Stahl and Schild (1969), using the same stationary phase and the same mobile phase. The reference solution was also the same as applied by Stahl and Schild (1969), or was composed of reference valepotriates, i.e. 20 mg of valtrate, 20 mg of acevaltrate and 20 mg of didrovaltrate in 10 ml of methanol. The plates were developed twice in a saturated chamber over a path of 15 cm. Under UV light of 254 nm valtrate and acevaltrate are visible as purple spots against a greenish fluorescent background. Spraying was done with a 22% antimony chloride solution, immediately followed by the 2,4-dinitrophenylhydrazine reagent. After 5 min at 105°C, valtrate, acevaltrate and IVHD turn blue and didrovaltrate becomes orange. The colour of the spots as well as the reagent are stable. Quantitative valtrate determinations were done directly on the thin-layer plate by using a densitometer (at 258.5 nm).

Verzár-Petri *et al.* (1976) applied two-dimensional TLC for the separation of valepotriates using hexane-methyl ethyl ketone 8:2, hexane-methyl ethyl ketone 9:1 and methylene chloride-methyl ethyl ketone 9:1 as mobile phases. Detection was done under UV light of 254 nm and after spraying with dinitrophenylhydrazine reagent.

Later, a better separation of the valepotriates was obtained by Hazelhoff *et al.* (1979b), using toluene-ethyl acetate-methyl ethyl ketone 80:15:5 as the mobile phase. This eluent was used for qualitative and quantitative TLC, in an unsaturated chamber, run 13 cm. Detection was achieved under UV light of 254 nm and again after spraying with dinitrophenylhydrazine reagent.

Rücker *et al.* (1981) described a TLC method by which valepotriates can be determined next to baldrinals. High-performance thin-layer chromatography (HPTLC) reversed phase (RP-C_{18}) F_{254} and silica gel 60 F_{254} plates (Merck, Darmstadt, Germany) were used. Mobile phases were methanol-water 8:2 for the reversed-phase plates, and methylene chloride-ethyl acetate-acetone 48:1:1 or methylene chloride-methyl ethyl ketone 98:2 for the straight-phase plates. Ammonia vapour was used as detection reagent. The reaction products of ammonia with valtrate, isovaltrate, acevaltrate, baldrinal and homobaldrinal could also be used for quantitative densitometric determinations. Valtrate and isovaltrate could be assayed without a detection reagent at 255 nm, the baldrinals without a detection reagent at 425 nm, and didrovaltrate after reaction with ammonia at 279 nm. Following exposure to ammonia vapour, valtrate and didrovaltrate could be assayed again, at 279 nm. This method was about ten-fold more sensitive than the detection with anisaldehyde reagent (Stahl and Schütz, 1980).

The reaction with NBP has been used by Braun *et al.* (1983) for the quantitative determination of valepotriates (see under 'Spectrophotometry'). In addition, the NBP reaction was shown to be useful for the detection of valepotriates by TLC. After separation of valerian root extracts on silica gel 60 F_{254} (Merck) using *n*-hexane-methyl ethyl ketone 8:2 as the mobile phase, the plates were sprayed with a 3% solution of NBP in acetone. After heating the plate at 40°C for 90 min, the plate was submerged in a 10% solution of tetrene in acetone. Valepotriates yield deep-blue to violet-blue spots.

- Gas chromatography (GC)

Verzár-Petri *et al.* (1976) described the first GC method for the determination of valtrate, acevaltrate and didrovaltrate. In addition, baldrinal could be detected. They used two different stationary phases, namely 2% OV-1 and 3% OV-17.

A GC determination of valepotriates has also been described by Graf and Bornkessel (1978). The substances, extracted by methylene chloride, were first separated using TLC. The valepotriates were then eluted from their respective zones, and hydrolysed with a 0.5 N methanolic potassium hydroxyde solution (60 min at 70°C). Each valepotriate yielded isovaleric acid that was subsequently extracted, after acidification, with diethyl ether containing propionic acid as the internal standard. Isovaleric acid was then determined by GC on a Carbowax column.

- High-performance liquid chromatography (HPLC)

The first HPLC method for the determination of valepotriates has been described by Tittel (1978), Tittel and Wagner (1978) and Tittel *et al.* (1978). They obtained a good separation suitable for qualitative as well as quantitative purposes from both crude material and preparations. A silica gel column with a particle size of 10 µm (MN-Nucleosil 50, 25 cm x 4 mm i.d., or Lichrosorb Si 100, 35 cm x 8 mm) and *n*-hexane-ethyl acetate 20:3 as the eluent were used. For detection, a refractive index and a UV detector were applied. The authors stated that this method was superior to the combined TLC-spectrophotometric method using the hydroxylamine-iron(III) chloride reagent (Wagner *et al.* 1970), with respect to time consumption, precision and sensitivity. The sequence of elution was valtrate and isovaltrate, didrovaltrate, acevaltrate.

For the determination of (iso)valtrate another HPLC method has been presented by Hazelhoff *et al.* (1979b). A Spherisorb Silica S5W column, 25 cm x 4.6 mm i.d., particle size 5 µm (Chrompack, Middelburg, The Netherlands), with 0.8% methanol in hexane as the mobile phase, and detection at 254 nm were applied.

Van Meer and Labadie (1981) described a straight-phase as well as a reversed-phase HPLC method for the analysis of valepotriates. The straight-phase column was a 5 µm Partisil silica column (Chrompack), 25 cm x 3 mm i.d. As eluents *n*-hexane-ethyl acetate 90:10 or 95:5, or *n*-hexane-ethanol 99.5:0.5 were used. The reversed-phase column was a 5 µm Spherisorb ODS (Chrompack), 25 cm x 3 mm i.d; methanol-water 50:50 or acetonitrile-water

60:40 were the eluents applied. Monoene valepotriates were detected at 206 nm, diene valepotriates at 256 nm.

Dossaji and Becker (1981) used reversed-phase HPLC with gradient elution for the determination of diene valepotriates. A Waters Bondapak C_{18} column, 30 cm x 3.9 mm i.d., was used with a methanol-water mixture as the eluent, A: 20:80 and B: 80:20; initially 85% B, finally 100% B in 5 min. Monoene valepotriates were separated on the same column, isocratic with methanol-water 80:20. Detection was done at 254 nm (dienes) and 208 nm (monoenes).

A comparable system was presented by Förster *et al.* (1984). The authors used an Altex Ultrasphere-ODS preparative column, 25 cm x 10 mm, particle size 5 µm, equipped with an Altex guard column (45 x 4.6 mm, 30 µm particles pellicular ODS). Monoenes were eluted with acetonitrile-water 70:30 and detected at 206 nm. For dienes a gradient was used of methanol-water mixtures, A: 60:40 and B: 90:10; initially 60% B, finally 90% B in 15 min. Detection of the dienes at 254 nm.

Chavadej *et al.* (1985) applied gradient elution for the monoenes as well as the dienes. A Lichrospher 100 CH (5 µm) column (Merck) was used. A 10 min gradient elution was achieved with methanol-water, A: 40:60 and B: 90:10; starting with 75% B to 95% B. The dienes were detected at 254 nm and the monoenes at 206 nm. As an internal standard *n*-pentylbenzene was used.

Valtrate and isovaltrate have been analysed in a methylene chloride extract of valerian, on a Lichrosorb RP-18 column (7 µm) using methanol-water 80:20 with 0.5% phosphoric acid as the eluent. Detection was done at 255 nm. With a flow rate of 2 ml/min, isovaltrate eluted after 6.4 min and valtrate after 7.4 min (Hänsel and Schulz, 1985).

Gränicher *et al.* (1992) applied the system of Förster *et al.* (1984), but with some modifications. They used a Nucleosil C_{18} column (25 cm x 4 mm i.d., 5 µm) fitted with a Nucleosil C_{18} guard column (30 x 4 mm i.d., 5 µm). Pentylbenzene was used as internal standard. The solvent system was methanol-water 90:10 (pump A) and methanol-water 40:60 (pump B). Elution of the diene type valepotriates was achieved isocratic with 60% A for 15 min, and a linear gradient to 90% A in 22 min. The monoene valepotriates were eluted with 60% A for 27 min, followed by a linear gradient to 80% A in 14 min.

Valerenic acid and derivatives

Initially, analytical procedures were focussed on the valepotriates as they were considered to be the main pharmacologically active constituents of valerian. In the 1980s, however, the valerenic acids started to attract attention, and analytical procedures for these compounds - only present in *V. officinalis* - were developed.

- Thin-layer chromatography (TLC)

For a better detection of acetoxyvalerenic acid in valerian root, Schimmer and Röder (1992) proposed to extend the TLC purity test described in the German Pharmacopoeia, DAB 9 (= procedure described in the European Pharmacopoeia 2nd edn (Anonymous, 1993c); see elsewhere in this chapter) with the TLC identity test for valerian tincture as included in the same pharmacopoeia (= procedure described in DAB 10; see elsewhere in this chapter), since acetoxy-valerenic acid could better be determined after conversion into hydroxyvalerenic acid.

In addition, Schimmer and Röder (1992) obtained a better separation of the zone due to valerenic acid from the tincture following a second development of the plate, using toluene-ethyl acetate 93:7.

In our laboratory the following TLC system is applied in order to check the identity of *V. officinalis*. An extract of 1 part of powdered plant material is made with 5 parts of ethanol [70%, v/v]. As a stationary phase, precoated channeled glass plates, silica gel, 250 µm layer (Baker Si250F, 19C) are used under the following conditions: unsaturated chamber; mobile phase, hexane-diethyl ether 6:4; distance of development, 15 cm; detection, spraying with 10 ml of anisaldehyde reagent followed by 5-10 min heating at 105°C. Samples of 10 µl of a 1% solution of the extract in methanol are applied. The detected compounds with their respective R_f values and the colours of the spots are listed in Table 2.

- High-performance liquid chromatography (HPLC)

Hänsel and Schulz (1982) were the first to describe an HPLC method for the determination of valerenic, acetoxyvalerenic and hydroxyvalerenic acids, and valerenal, in both the crude drug and tinctures of *V. officinalis*. The co-occurrence of the sesquiterpenoids valerenic and acetoxyvalerenic acid, and the possible presence of hydroxyvalerenic acid, is confined to *V. officinalis*; this

Table 2. Characteristic thin-layer chromatogram of the main components of *V. officinalis*

Compound	R_f	Colour
Hydroxyvalerenic acid	0.01	Violet
Acetoxyvalerenic acid	0.15	Violet
Valerenic acid	0.38	Violet
Baldrinal	0.47	Yellow
Cryptofauronol	0.65	Purple-violet
Patchouli alcohol	0.70	Brownish-blue
Valerenal	0.86	Blue
Valeranone	0.89	Yellow

HPLC method can therefore be employed to detect adulterations by non-officinal valerian root extractives (like *V. wallichii* and/or *V. edulis*).

The HPLC method consisted of a reversed-phase analysis using an RP-18 (7 µm) column (Knauer, Berlin, Germany), 25 cm x 4.6 mm i.d., and a guard column of 40 mm. A mixture of methanol-water 80:20 with 0.5% phosphoric acid was used as the eluent (pH 2). Detection was done at 225 nm. It was shown that valepotriates had retention times that were comparable with those of the sesquiterpenoids, disturbing the analysis of root material. The lipophilic acids were therefore separated from the lipophilic neutral components by treatment with alkali. After acidification of the alkaline aqueous fraction containing the sesquiterpenoids, they were extracted with chloroform. Tinctures of *V. officinalis* were analysed directly, as they were devoid of valepotriates. Biphenyl was used as an internal standard.

Freytag (1983) presented a simplified and more reproducible procedure when compared with the method of Hänsel and Schulz (1982). The crude root material (*V. officinalis*) was extracted with methylene chloride; the solvent was evaporated, and the residue dissolved in methanol. This extract contained both valerenic acids and valepotriates. The same procedure, but with ethanol-water 70:30 with 1% acetic acid as extraction solvent yielded an extract free of valepotriates, whereas the valerenic acids were quantitatively extracted. HPLC was done using a Lichrosorb RP-18 (5 µm) column, 25 cm x 4.6 m i.d., and acetonitrile-phosphoric acid (pH 2.0) 65:35 as the mobile phase. The compounds were detected at 225 nm. Using this procedure, acetoxyvalerenic acid was determined as such, instead of in the form of hydroxyvalerenic acid. No alkaline-

acid liquid-liquid extraction was necessary to separate the valerenic acids from the valepotriates.

Recently, Gobbato and Lolla (1996) described an HPLC method for the analysis of valerenic acids in *V. officinalis* extracts using a Zorbax SB-C_{18} column, 25 cm x 4.6 mm i.d., 5 µm (Rockland Technologies, Inc., Newport, USA). For elution, an acetonitrile-phosphoric acid [0.1%, v/v] gradient was used. The column temperature was 40°C, and the detection was at 225 nm.

Baldrinals

- Thin-layer chromatography (TLC)

A TLC method for the determination of baldrinal and homobaldrinal - next to valepotriates - was described by Rücker *et al.* (1981); details are mentioned above under 'Valepotriates'.

Essential oil

In order to meet the definition of *Valerianae radix* given in the European Pharmacopoeia 3rd edn (Anonymous, 1997), an assay is carried out according to the determination of essential oils in vegetable drugs. This is a hydrodistillation in a special apparatus; the oil is collected in a graduated tube, while the aqueous phase is recirculated into the distillation flask. Using the assay conditions prescribed, the crude drug should contain not less than 0.5% (v/w) of essential oil.

A description of the essential oils of *V. officinalis* and *V. wallichii* is given in The Wealth of India (Anonymous, 1976). The oil from *V. officinalis* roots is olive-green to olive-brown in colour. It has a warm-woody, balsamic-root-like odour with a distinct animal undertone of musk-like character and great tenacity. A fresh-green, slightly camphoraceous top-note is also typical in the odour of good oil. Under poor storage conditions and on ageing, the oil turns darker, becomes more viscous, and acquires an objectionable odour of valeric and isovaleric acids. The oil from *V. wallichii* roots is a pale-brown or amber-yellow coloured liquid with a root-like odour and with a distinct note of valeric acid, more or less pronounced according to the age of the oil. Musk-like and patchouli-like camphoraceous notes are quite characteristic. The oil distilled from shade-dried roots, not too old, has a finer odour reminiscent of violet leaf oil. The odour and flavour of *V. wallichii* oil is considered poor as compared to

V. officinalis oil. The chemical composition of the essential oils of the *Valeriana* species of interest will be discussed in the following chapters.

Storage conditions

According to the European Pharmacopoeia 3rd edn (Anonymous, 1997), *Valerianae radix* should be stored in a well-closed container, protected from light.

Powdered valerian root rapidly loses its essential oil and will no longer comply with the pharmacopoeial standard. In addition, the drying as well as the storage temperature are important. At temperatures higher than 40°C, the valepotriates start to decompose, yielding valeric and isovaleric acids. The characteristic odour of these acids points to material dried or stored improperly. Additionally, hydroxyvalerenic acid may be considered to be a decomposition product of acetoxyvalerenic acid when the drug is stored at a too high humidity (Freytag, 1983; Bos *et al.* 1996).

Valerenic acid and its derivatives have been proved to be stable in both plant material and preparations. In contrast, valepotriates rapidly decompose if water is present. They show a temperature-dependent instability. In commercially available valerian tinctures, for instance, valepotriates could not be detected anymore. After a storage time of several weeks these compounds have decomposed, first yielding baldrinals, that may react further with yet unknown substances present in the tincture or form polymerization products (Bos *et al.*, 1996).

Regulatory aspects

Present situation

For quality control of *Valeriana radix* and of phytomedicines prepared from valerian, the monograph of the European Pharmacopoeia 3rd edn (Anonymous, 1997) can be used, but additional analyses are necessary (Woerdenbag, 1995).

Between various countries of the European Union and in the United States of America, large differences exist in the legal position of herbal medicines. Regulations vary considerably from country to country, and in most European countries and in the USA the legislation of herbal drugs is much les progressive than that of synthetic drugs.

The German health authorities, 'Bundesinstitut für Arzneimittel und Medizinprodukte' (formerly 'Bundesgesundheitsamt'), have set up an expert committee for the evaluation of herbal drugs, the so-called 'Kommission E'. The findings of the Kommission E, based on data available from the literature, are laid down in a monograph, in which the balance between usefulness and risk is weighed (Woerdenbag *et al.*, 1993; Woerdenbag, 1995). For valerian a 'Positiv-Monographie' exists, meaning that the herbal drug has been found to be biologically active, without inducing serious side effects (Anonymous, 1992a). The root is permitted for oral use, and for preparation of herbal tea and tincture (De Smet, 1993).

In France and Belgium, herbal remedies are subject to general drug regulations, and should therefore comply with criteria of efficacy, safety and quality. As compared with synthetic drugs, a simplified admission procedure is applied, which is based on chemical and pharmaceutical documentation, as available from the literature. Subterranean parts of *V. officinalis* and preparations therefrom are permitted for oral use in France. In Belgium subterranean parts, powder, extract and tincture are permitted as traditional tranquillizer (De Smet, 1993).

In the United Kingdom, there are no special guidelines for the admission of herbal remedies to the drug market. To be accepted as a medicinal product, a herbal preparation must comply with the Guidelines on Safety and Efficacy Requirements for Herbal Medicinal Products. As proofs of safety and efficacy, literature data should be submitted. For acceptance as an approved drug, however, evidence from clinical trials is required. For this reason, only few phytomedicines, not including valerian, have reached the status of approved drug in the United Kingdom (De Smet, 1993).

In The Netherlands, no other legal regulations than the European Pharmacopoeia 3nd edn (Anonymous, 1997) and the 'Warenwet' (Food Act) exist for phytomedicines, which do not have the status of drugs therefore, and may not be called a drug or recommended as such. In principle, herbal preparations can be sold by anyone in The Netherlands, a situation which tends to provoke inexpert use (Woerdenbag, 1995).

It is clear that there is no unity at all in the European Union, in the field of herbal drugs. In 1989 the European Scientific Cooperative on Phytotherapy (ESCOP) was founded. The general aim of ESCOP is to advance the scientific status of phytomedicines and to assist with harmonization of their regulatory

status in European countries. In a European frame-work, ESCOP prepares monographs for herbal drugs. These monographs, officially known as SPCs (Summary of Product Characteristics), are offered to regulatory authorities, the Committee on Proprietary Medicinal Products (CPMP), as a means of harmonizing the medicinal uses of plant drugs in the European Union and in a wider European context. An SPC of *Valerianae radix* has been submitted to the CPMP (Krant, 1994).

ESCOP defines phytomedicines as follows: 'Phytomedicines (plant medicines) are medicinal products containing as active ingredient only plants, parts of plants or plant materials, or combinations thereof, whether in the crude or processed state'.

Many of today's widely used herbs were once the subject of monographs in The United States Pharmacopoeia and The National Formulary, including valerian root (*V. officinalis*). No such legal standards exist in the USA today. The Food and Drug Administration (FDA) has evaluated the safety and efficacy of several herbal medicines. Valerian root has been granted the 'Generally Recognized As Safe' (GRAS) status (De Smet, 1993; Tyler, 1993; 1994).

The future

In the near future, the European Pharmacopoeia Commission is expected to change the monograph on valerian root. An HPLC assay for the quantitative determination of valerenic acid will be included, as well as a demand for its content in the crude drug. The present demand of at least 0.5% (v/w) essential oil will then probably be abandoned (J.H. Zwaving, personal communication). The quantitative determination of valerenic acid and derivatives insures that extracts are not adulterated by roots of *V. wallichii* and/or *V. edulis* (Hänsel and Schulz, 1985).

Hänsel and Schulz (1985) proposed criteria for the content of valerenic acid and its derivatives. They recommended that alcoholic extracts of *V. officinalis* should contain valerenic acids with a minimum of 120 mg/100 g and aqueous extracts a minimum of 60 mg/100 g.

Valerian based phytomedicines are sometimes standardized (Bos *et al.*, 1996). Preferably this should be done on valerenic acid and its derivatives, because of their stability. This implies that *V. officinalis* is the crude material of choice for such phytomedicines (Hänsel, 1992).

For the European Pharmacopoeia a monograph on *Extractum Valerianae siccum* (dry valerian extract) is in preparation (Zwaving, 1993).

Pharmacology and uses

Valerian root has already been used by the Greek and the Roman physicians as a diuretic, anodyne and spasmolytic agent. In the 17th century it was used to treat epilepsy. Its current use as a mild sedative dates back to the 18th century (Stoll and Seebeck, 1957; Madaus, 1976). Valerian has often been used in conjunction with bromides, chloral hydrate, and phenobarbital, in the treatment of hysteria and other nervous conditions. It has also been used as a carminative (Reynolds and Prasad, 1982). Valepotriates have been applied to influence psycho-vegetative and psychosomatic disorders in cases of restlessness, agony and tension, as well as lack of concentration (Steinegger and Hänsel, 1992).

Nowadays, valerian preparations are used primarily to treat light forms of neurasthenia and emotional stress (Anonymous, 1993a). Further indications are disturbances in falling asleep and cramping pains in the gastro-intestinal tract, as a consequence of tension (Steinegger and Hänsel, 1992). *V. wallichii* is used in Ayurvedic medicine (Kapoor, 1990), e.g. as a sedative.

In the past decades numerous studies have been directed to the pharmacology of valerian extracts and their isolated constituents, as reviewed by Houghton (1988). Originally, the essential oil and especially its constituents bornyl acetate and bornyl isovalerate were held responsible for the mild sedative properties of valerian preparations (Gstirner and Kleinbauer, 1958), but later this did not appear to account for the entire action of the extract. Although several alkaloids from valerian root have been found to exhibit sedative effects in rats (Rabbeno, 1930; Borkowski and Lutomski, 1961; Cionga, 1961; Sándor *et al.*, 1970), they are unlikely to play a significant role in the biological activity of valerian preparations because of their very low concentrations. Aqueous extracts of valerian roots only showed a sedative effect in laboratory animals at extremely high doses (Hänsel, 1990).

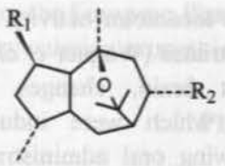
After the characterization and demonstration of the biological activity of the valepotriates in the late 1960s and early 1970s, many investigators focussed their attention on these substances. A mixture of various valepotriates, as present in an extract of *V. wallichii*, was found to have sedative properties in mice (Von Eickstedt and Rahman, 1969). In addition, a spasmolytic effect of valepotriates on smooth muscle of the guinea pig was described (Wagner and Jurcic, 1979;

Hazelhoff *et al.*, 1982). A decrease of spontaneous locomotor activity of mice has also been noticed after administration of valepotriates (Wagner *et al.*, 1980; Hölzl and Fink, 1984). In the perfused rat brain, changes in the electroencephalogram (EEG) have been found which were induced by valepotriates (Fink *et al.*, 1984). However, following oral administration of valepotriates, only a small fraction is absorbed. Therefore, no evidence exists that pharmacologically relevant concentrations are reached in the central nervous system after oral ingestion (Steinegger and Hänsel, 1992). In a neurophysiological study with cats that were given valepotriates or valerian extract orally, no changes in the EEG were observed that could point to a central sedative effect. The muscle tonus of treated animals, however, became reduced (Holm *et al.*, 1980). It has been concluded by Grusla *et al.* (1986) and Krieglstein and Grusla (1988) that valepotriates, valerenic acid, valeranone as well as the essential oil of valerian do not possess a central depressant action, because these substances were not found to induce a reduction of the glucose turnover in the rat brain.

It has been demonstrated, in the late 1950s and early 1960s, that valerenic acid possesses spasmolytic properties and that valeranone has anticonvulsive, hypotensive and sedative effects (Stoll *et al.*, 1957a; Arora and Arora, 1963; Rücker *et al.*, 1978). Furthermore, the related species *Nardostachys jatamansi* DC., which was used in Asia for the treatment of nervous diseases, was shown to contain valeranone but lacked valepotriates. Later, the pharmacological properties of valeranone were confirmed, and also valerenal and valerenic acid were found to show central depressant and spasmolytic properties (Hendriks *et al.*, 1981a; 1985).

In the so-called syndrome test, in which a series of symptoms are observed in mice after administration of a test compound, a general depressant effect was found for several constituents of the essential oil of *V. officinalis* (Hendriks *et al.*, 1981a). For valerenic acid, non-specific central depressant effects have been described following intraperitoneal administration to mice. At doses above 100 mg/kg body weight, effects were found in a rotarod test and in a traction test. Spontaneous locomotor activity of mice was reduced by valerenic acid at a dose of 50 mg/kg. At this dose a prolongation of the barbiturate-induced sleeping time was found as well (Hendriks *et al.*, 1985).

Studies carried out in Japan showed that the two monoacetates (**27a** and **27b**) of kessoglycol (**26**) as well as its diacetate (**27c**), compounds that were also isolated from valerian roots, enhanced a hexobarbital-induced anaesthesia (Takamura *et al.*, 1975a; 1975b; Hikino *et al.*, 1980). The activity of the diacetate



26 Kessoglycol
$R_1 = R_2 = OH$
27a Kessoglycyl 2-acetate
$R_1 = OCOCH_3$, $R_2 = OH$
27b Kessoglycyl 8-acetate
$R_1 = OH$, $R_2 = OCOCH_3$
27c Kessoglycyl diacetate
$R_1 = R_2 = OCOCH_3$

was assumed to be due to its inhibitory effect on the central nervous system; the compound did not exhibit an inhibitory action on stress-induced ulcer formation.

Recently, a commercially available root extract prepared from *V. officinalis* and standardized on valerenic acid was shown to produce a moderate, dose-related sedation in mice following oral administration. Reduction in motility and an increase of the thiopental-induced sleeping time were used as parameters, and the extract was compared with diazepam and chlorpromazine. The extract showed only weak anticonvulsive properties (Leuschner *et al.*, 1993).

Valerian root essential oil and some volatile compounds (borneol, isoborneol, bornyl acetate and isobornyl acetate) are also used in aromatherapy. It has been suggested that a sedative effect may arise from inhalation of the monoterpenes, but the only evidence for this comes from animal experiments (Buchbauer *et al.*, 1992).

In several clinical studies good results have been reported for various valerian preparations, including mild sedation, decreased perceived sleep latencies and awakenings, and improved sleep (Buchthala, 1968; Dziuba, 1968; Standl, 1968; Straube, 1968; Hübler, 1969; Jauch, 1969; Jansen, 1977; Leathwood *et al.*, 1982; 1983; Lindahl and Lindwall, 1989; Dreßing *et al.*, 1992). However, Hölzl (1996) recently reviewed clinical studies with valerian extracts and emphasized that, in general, the quality of these studies was suboptimal, and that the exact chemical composition of the preparations used was often not clear.

Despite the fact that rather extensive research has been conducted on valerian, it is still unclear which compound(s) is (are) responsible for its sedative action (Houghton, 1988; Bos *et al.*, 1996). It has been suggested that a combination of constituents is responsible for the effect and that degradation products of genuine constituents could also play a role (Weiß, 1990). However, it has been concluded by Hazelhoff (1984) that the reputed tranquillizing effect of valerian preparations could be totally or primarily due to a peripheral effect (viz. spasmolysis) rather than to a real central effect (Grusla *et al.*, 1986).

A pharmacological review about extracts and compounds of the three valerian species used in phytomedicines is given by Reichling *et al.* (1994).

Adverse effects

No significant adverse reactions or side effects have been reported as a result of normal medication with valerian drugs (Hänsel, 1990; Houghton, 1994). The same was found in the clinical studies performed with valerian extracts, so far. The acute toxicity of valerian preparations is considered to be very low. However, studies directed to subchronic and chronic toxicity are still lacking and such studies are certainly warranted, especially because valepotriates and their degradation products, the baldrinals, may be capable of causing undesired effects. In particular when valerian preparations are taken for a longer period of time, the absence of chronic risk data should be considered.

Recently a review of literature data on a number of possible adverse effects of valerian and of compounds isolated from valerian roots was published (Bos *et al.*, 1997a). Some examples will be given here.

Willey *et al.* (1995) treated a young woman with activated charcoal after she had ingested about 20 g of valerian roots in a suicide attempt. Her clinical course was benign, with no other symptoms than fatigue, abdominal cramping, chest tightness, tremor of the hands and feet, lightheadedness and mydriasis.

Valerenic acid, valeranone, and the essential oil and extracts from valerian root have been shown to increase the barbiturate-induced sleeping time in mice (Von Eickstedt and Rahman, 1969; Sándor *et al.*, 1970; Shrivastava and Sisodia, 1970; Takamura *et al.*, 1973; Rücker *et al.*, 1978; Hendriks *et al.*, 1985). This may indicate that the effect of central nervous depressants is amplified by valerian constituents.

One double-blind study with volunteers has been directed to a possibly synergistic effect of the simultaneous consumption of valepotriates and alcohol. Oral administration of a mixture of valepotriates (valtrate, acevaltrate and didrovaltrate, 200-400 mg) was followed by a dose-dependent increase in the ability to concentrate. In combination with ethanol an expected reduction of efficiency was not observed. Valtrate, tested as a separate compound, did not influence the height and course of the blood alcohol curves (Mayer and Springer, 1974).

Valerian preparations are considered safe for use during pregnancy and lactation (Berglund *et al.*, 1984; Anonymous, 1993a). According to an Australian

Government Publishing Service publication valerian is placed in category A: Drugs which have been taken by a large number of pregnant women and women of childbearing age without an increase in the frequency of malformations or other direct or indirect harmful effects on the fetus having been observed (Anonymous, 1992c).

Valerian-containing products have at times been associated with hepatotoxic reactions but such products contained other herbal ingredients which could have been responsible (notably skullcap) (McGregor *et al.*, 1989; Miskelly and Goodyer, 1992; Shepherd, 1993). However, mutagenic effects on the liver by valepotriates and baldrinals cannot be excluded, as is indicated in the following paragraph.

The valepotriates possess alkylating properties, for which the epoxy group is responsible. Cytotoxic properties against *in vitro* cultured tumour cells have been described for valepotriates. The mechanism of the cytostatic action was supposed to be based on an interaction of the valepotriates with thiol-containing enzymes (Becker and Chavedej, 1988). The cytotoxic effects of the valepotriates on cultured hepatoma cells were counteracted by compounds with free SH groups, such as cysteine and glutathione (Keochanthala-Bounthanh *et al.*, 1990). In addition, valepotriates inhibited the synthesis of DNA and proteins (Keochanthala-Bounthanh *et al.*, 1993). Cytotoxic properties are also known for baldrinals (Bounthanh *et al.*, 1983; Dieckmann, 1988; Keochanthala-Bounthanh *et al.*, 1993).

It is unclear to what extent these toxic effects are relevant for humans after ingestion of valepotriate-containing preparations. As already stated, valepotriates are hardly absorbed in their original form. In the gastro-intestinal tract, baldrinals and possibly polymers are formed from the valepotriates. Subsequently, the baldrinals are rapidly glucuronidated in the liver. The metabolites that originate at this point, are not mutagenic (Hänsel, 1992). However, due to the contact of the valepotriates and baldrinals with the stomach and intestinal wall, the gastro-intestinal tract and the liver are the primary target organs which may be exposed to mutagenic effects (Dieckmann, 1988). This may especially be true when valerian preparations are taken over a longer period of time, as is done quite often (Hänsel, 1990). As yet, there is a lack of long-term studies on this subject. Therefore, it would be prudent to prefer valerian preparations which are devoid of the potentially hazardous valepotriates or baldrinals (De Smet and Vulto, 1988; Hänsel, 1992).

Chapter 2

ISOLATION AND IDENTIFICATION OF VALERIAN COMPOUNDS[2]

Introduction

For analytical purposes it was necessary to isolate sufficient amounts of the reference compounds valerenic acid, hydroxyvalerenic acid and acetoxyvalerenic acid from the roots and rhizomes of *Valeriana officinalis* L. *s.l.*, because these compounds are not commercially available.

After several pilot experiments we started an extraction and isolation procedure based on the presence of the COOH function in the valerenic acids. This makes it possible to separate the acids as their sodium salts from other, lipophilic, constituents present in the original (methylene chloride) extract. One of these compounds is valerenal, the corresponding aldehyde of valerenic acid. This compound was isolated because it may also contribute to the biological activity of valerian roots (Hendriks *et al.*, 1981a).

For the isolation and purification of the compounds we used liquid column chromatography (LC), preparative thin-layer chromatography (TLC) and gas chromatography (GC); fractions obtained from the chromatographic purification steps were monitored by TLC, GC and gas chromatography-mass spectrometry (GC-MS).

A large number of known compounds could be detected in several fractions, particularly mono- and sesqui-terpenoids. In addition we isolated a number of unknown compounds of which the structure could be elucidated by

[2]This chapter is based on:
Bos R, Hendriks H, Kloosterman J, Sipma G (1983) A structure of faurinone, a sesquiterpene ketone isolated from *Valeriana officinalis*. *Phytochemistry* **22**, 1505-1506.
Bos R, Hendriks H, Bruins AP, Kloosterman J, Sipma G (1986) Isolation and identification of valerenane sesquiterpenoids from *Valeriana officinalis*. *Phytochemistry* **25**, 133-135.
Bos R, Hendriks H, Kloosterman J, Sipma G (1986) Isolation of the sesquiterpene alcohol (-)-pacifigorgiol from *Valeriana officinalis*. *Phytochemistry* **25**, 1234-1235.
Bos R, Woerdenbag HJ, Hendriks H, Kruizinga WH, Herrema JK, Scheffer JJC (1997) Methyl valerenate, a new sesquiterpenoid in the essential oil from underground parts of *Valeriana officinalis* L. *s.l.* *Z. Naturforsch.*, **52c**, xxx-xxx.

means of ultraviolet (UV), infrared (IR), and ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectroscopy, and mass spectrometry (MS).

In the course of these phytochemical investigations it was decided also to use the essential oil as starting material for the isolation of some of the unknown constituents, facilitating some of the purification steps.

In this chapter we describe general procedures for the extraction and distillation of the plant material, and for the fractionation of the extract and the essential oil. More detailed experimental information is given in the separate paragraphs concerning the compounds in question.

General experimental procedures

Plant material

A commercial sample of roots and rhizomes of *V. officinalis* L. *s.l.* was obtained from the Verenigde Nederlandse Kruidencoöperatie ua, Elburg, The Netherlands. A voucher specimen has been deposited at the Department of Pharmaceutical Biology, University Centre for Pharmacy, Groningen, The Netherlands.

Extraction of the plant material

Dried, powered roots (30 kg) of *V. officinalis* were percolated for 3 days with about 100 l of methylene chloride. The extract was concentrated to 3 l and then extracted twice with the same volume of an aqueous 2% NaOH solution. The combined aqueous layers were acidified with HCl and extracted twice with 2 l of petroleum ether-diethyl ether 2:1, giving a solution of the organic acids (solution A). The methylene chloride extract remaining after the extraction with the NaOH solution, was washed with water until neutral, dried over anhydrous sodium sulphate, and concentrated by means of a rotary evaporator. The residue was redissolved in 3 l petroleum ether and filtered (solution B).

Isolation of the essential oil

Dried roots and rhizomes (5 kg) of *V. officinalis* were submitted to steam distillation following the method of Hendriks (1973) and yielded 0.55% (v/w) essential oil. This procedure was repeated several times.

Fractionation of the essential oil

The essential oil was divided into fractions with hydrocarbons (mono- and sesquiterpenes) and oxygen-containing compounds (e.g. mono- and sesquiterpenoids) by means of column chromatography over silica gel 60 (60-230 mesh; Merck, Darmstadt, Germany) with petroleum ether (b.p. <40°C) and diethyl ether as eluents, respectively (Hazelhoff *et al.*, 1979a).

Gas chromatography (GC)

GC analysis was performed on a Packard Instruments 430 gas chromatograph under the following conditions: column, WCOT fused-silica CP-Sil 5 (25 m x 0.32 mm id., film thickness, 0.25 μm; Chrompack, Middelburg, The Netherlands); oven temperature programme, 60-250°C at 4°C/min; injector temperature, 250 °C; detector (FID) temperature, 300 °C; carrier gas, nitrogen; inlet pressure, 5 psi; split ratio 50:1; injected volume, 1.0 μl; and on a Hewlett-Packard 5890 Series II gas chromatograph equipped with a 7673 injector and a Hewlett Packard 3365 Series II Chemstation, under the following conditions: column, WCOT fused-silica CP-Sil 5 CB (25 m x 0.32 mm i.d., film thickness, 0.25 μm; Chrompack); oven temperature programme, 50-290°C at 4°C/min; injector and detector (FID) temperatures as above; carrier gas, nitrogen; inlet pressure, 5 psi; linear gas velocity, 26 cm/s; split ratio, 56:1; injected volume, 1.0 μl (for analysis of compound **29**).

Gas chromatography-mass spectrometry

GC-MS (EI) was performed on a Finnigan 9500/3300/6110 GC-MS computerized system, modefied for negative ion operation (Bruins, 1983). The GC conditions were: column, WCOT fused-silica CP-Sil 5 (25 m x 0.25 mm i.d., film thickness, 0.25 μm; Chrompack); oven temperature programme, 60-300°C at 6°C/min; injector temperature, 250°C; carrier gas, helium; inlet pressure, 5 psi; flow rate, 1.5 ml/min; linear gas velocity, 30 cm/s; split ratio, 12:1. MS conditions: ionization energy, 70 eV; ion source temperature, 185°C; interface temperature, 290°C; scan speed, 1 scan/s; mass range, 34-500 u. The reactant gas mixture for negative ion chemical ionization (NICI) was ca CH_4-N_2O, 1:1 (Smit and Field, 1977; Bruins, 1983).

Preparative gas chromatography

F & M 700 and F & M 720 gas chromatographs with, katharometer detectors were used under the following conditions:

- Column 1, 3% OV-1 on Chromosorb W-HP, 80-100 mesh (stainless steel, 2 m x 2 cm i.d.); oven temperature, 200°C; injector and detector temperatures, 250°C; carrier gas, helium; flow rate, 25 ml/min;
- Column 2, 10% Carbowax 20M on Chromosorb G-HP, 80-l00 mesh (stainless steel, 2 m x 2.3 cm i.d.); oven temperature programme, 80-200°C at 4°C/min; injector and detector temperatures, 250°C; carrier gas, nitrogen; flow rate, 30 ml /min.

Nuclear magnetic resonance (NMR) spectroscopy

^{1}H- (99.55 MHz) and ^{13}C-NMR (25.0 MHz) data of the components **11-17** were recorded on a Jeol FX-100 spectrometer. Deuterochloroform was used as solvent, with TMS as internal standard.

^{1}H- and ^{13}C-NMR (APT) data of the components **6-8** and **19** were recorded on a Varian-500 Unity spectrometer (500 and 125.72 MHz). Chemical shifts are denoted in δ-units (ppm) relative to the solvent ($CDCl_3$) and converted to the TMS scale. The splitting patterns are designated as follows: s (singlet), d (doublet), dd (double doublet), t (triplet), q (quartet), m (multiplet) and br (broad). Chemical shifts (in ppm) are positive in lowfield direction. When references are made towards the text, designation follows (multiplicity, signal form, number of protons, number in the text).

Before measuring the 2D spectra, the exact 90° pulse width was determined. The shift-correlated 2D NMR (COSY), incoherence transfer (NOESY) and C-H correlation by polarization transfer (HETCOR) spectra were recorded using standard Varian microprogrammes.

Thin-layer chromatography (TLC)

Silica gel plates (20 x 20 cm, 0.25 mm, G1500 LS 254, Schleicher & Schüll, Dassel, Germany) were used for monitoring the fractions. Preparative silica gel plates (20 x 20 cm, 1 mm, G1510 LS 254 (Schleicher & Schüll) were used for the isolation of several compounds.

Optical rotation

Optical rotation was measured on a Perkin-Elmer 241 polarimeter.

Ultraviolet and Infrared (UV and IR) spectroscopy

UV and IR spectra were obtained with a Spectroflex III (Pharmacia) spectrophotometer and an ATI Mattson Genesis Series FTIR spectrometer, respectively.

Isolation of cyclopentane sesquiterpenoids

Isolation of valerenic, hydroxyvalerenic and acetoxyvalerenic acids

After evaporation of the petroleum ether-diethyl ether mixture, solution A yielded about 500 g of a residue which was redissolved in 1 l of petroleum ether and stored at -20°C for 24 h. The precipitate collected by filtration contained ferulic, valerenic and hydroxyvalerenic acids. This precipitate was submitted to column chromatography (silica gel 60, 40 x 4 cm) using petroleum ether-diethyl ether mixtures from 10% up to 100% diethyl ether in 10% steps. The 20% diethyl ether fraction contained valerenic acid, while ferulic acid and hydroxyvalerenic acid were obtained after elution with 100% diethyl ether. The latter compounds were isolated by preparative TLC (petroleum ether-diethyl ether 2:8). Acetoxyvalerenic acid was obtained by preparative TLC of the remaining petroleum ether solution using petroleum ether-diethyl ether 6:4.

Isolation of valerenal, valerenol and some valerenyl esters

After evaporation of the solvent from solution B, 500 g of a residue containing the terpenoid compounds were obtained. The residue was diluted with 500 ml of petroleum ether and transferred onto a silica gel column (125 x 12 cm). Column chromatography was performed using 20 l of petroleum ether, 50 l of petroleum ether- diethyl ether mixtures 99:1 to 90:10 (in 1% steps), 5 l of petroleum ether-diethyl ether 3:1, 5 l of petroleum ether-diethyl ether 1:1 and 10 l of diethyl ether.

Fractions of about 1 l each were collected and, after GC analysis, the fractions 26-34 mainly containing the valerenyl esters, were combined, concentrated and submitted to EI- and NICI-GC-MS (Smit and Field, 1977; Bruins, 1979). *Z*-Valerenyl acetate and *E-/Z*-valerenyl isovalerate were isolated by means of preparative TLC (petroleum ether-diethyl ether 8:2) followed by preparative GC, using column 1. The fractions 36-39 mainly contained valerenal and valerenol. These components were isolated by preparative GC, using column 1.

Isolation of faurinone

About 1 g essential oil was divided into several fractions by column chromatography over silica gel 60 (1 m x 4 cm) using petroleum ether containing an increasing percentage of diethyl ether (0-100%). The eluated fraction 98:2 (115 mg) contained valeranone (55.6%), valerenal (20.0%), a number of valerenyl esters (9.6%) and a sesquiterpene ketone, $C_{15}H_{26}O$ (10.4%). This ketone was isolated by preparative GC, using column 2. The last procedure was repeated several times to obtain about 150 mg of the ketone.

The spectral data, IR, ^{1}H-NMR and mass spectra, were identical with those of faurinone (**22a**), first reported by Hikino *et al.* (1968). However, the ^{13}C-NMR spectrum of our isolated compound revealed the presence of only one quaternary carbon atom, which was not in accordance with structure **22a**.

Synthesis and isolation of faurinol isomers

A sample of 110 mg faurinone in 0.5 ml of dry diethyl ether was added to 10 mg $LiAlH_4$ in 1 ml of dry diethyl ether. After 10 min, a few drops of ethyl acetate and 5 ml water were added to the solution, which was poured into 10 ml of H_2SO_4 [2%]. After extraction with diethyl ether (3 x 10 ml) and drying over anhydrous sodium sulphate, 73 mg of faurinol isomers were obtained. The two isomers were separated by preparative TLC with petroleum ether-diethyl ether 7:3 as mobile phase.

Isolation of pacifigorgiol

A sesquiterpene alcohol was isolated from the fractions 41-45 that were obtained by fractionation of the residue from solution B, as described above; it proved to be a tertiary alcohol with a molecular formula $C_{15}H_{26}O$. The percentage of this compound in the essential oils of different *V. officinalis* strains varied from 0.7% to 8.6%.

Low-pressure column chromatography using silica gel 60 (125 x 4 cm) and a petroleum ether-diethyl ether mixture 6:4 gave about 250 mg of an amorphous substance. Preparative TLC (petroleum ether-diethyl ether 5:5) yielded 175 mg of pure pacifigorgiol.

Synthesis of methyl valerenate

Valerenic acid, 2.1 mmol, was dissolved in 10 ml of diethyl ether and treated with 27 ml of a solution of diazomethane in diethyl ether (0.76 mmol/ml). After

evaporation of the solvent and the reagent, 2.0 mmol methyl valerenate was obtained as a colourless fluid.

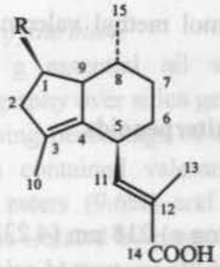
Identification of cyclopentane sesquiterpenoids

Valerenic acid (**1**)

$[\alpha]^{20}_D$: -117.8° (c = 1.64, EtOH); UV (EtOH) λ_{max} (log ε) 218 nm (4.232); IR ν (KBr, max.) cm^{-1} : 3600-2400 (COOH), 1675 (C=O, α,β-unsaturated acid), 1630 (C=C); EI-GC-MS *m/z* (rel. int. %): 235 $[M+1]^+$ (12), 234 $[M]^+$ (71), 189 (34), 161 (81), 148 (39), 147 (41), 133 (75), 122 (79), 121 (38), 119 (42), 117 (30), 107 (96), 105 (82), 95 (39), 93 (52), 91 (100), 81 (36), 79 (54), 77 (51), 55 (46), 41 (69), 39 (34); ^{1}H-NMR: δ 7.18 (dq, $^2J_{11,5}$= 9.7 Hz, $^4J_{11,13}$= 1.5 Hz, 1H, H-11), 3.55 (dd, $^3J_{5,11}$= 9.7 Hz, $^4J_{5,6}$= 4.7 Hz, 1H, H-5), 2.95 (m, 1H, H-9), 2.20 (bt, $^3J_{2,1}$= 7.7 Hz, 2H, H-2), 1.99 (m, 1H, H-8), 1.89 (d, $^4J_{11,13}$= 1.5 Hz, 3H, H-13), 1.86 (m, 2H, H-7), 1.82 (m, 2H, H-1), 1.78 (m, 2H, H-6), 1.64 (t, $^4J_{10,2}$= 0.9 Hz, 3H, H-10), 1.56 (m, 2H, H-1), 1.42 (m, 2H, H-7), 1.39 (m, 2H, H-6), 0.79 (d, $^3J_{15,8}$= 7.0 Hz, 3H, H-15); ^{13}C-NMR: δ 174.4 (C=O, C-14), 146.2 (CH, C-11), 133.0 (C, C-4), 131.1 (C, C-3), 125.1 (C, C-12), 47.2 (CH, C-9), 37.2 (CH_2, C-2), 34.4 (CH, C-5), 32.8 (CH, C-8), 28.5 (CH_2, C-7), 25.1 (CH_2, C-6), 24.3 (CH_2, C-1), 13.3 (CH_3, C-13), 11.8 (CH_3, C-15), 11.7 (CH_3, C-10).

Hydroxyvalerenic acid (**2**)

$[\alpha]^{20}_D$: -98.4° (c = 0.63, EtOH); UV (EtOH) λ_{max} (log ε) 212 nm (4.305); IR ν (KBr, max) cm^{-1}: 3600-2400 (COOH), 3340 (OH), 1676 (C=O, α,β-unsaturated acid), 1635 (C=C); EI-MS (direct inlet) *m/z* (rel. int. %): 250 $[M]^+$ (8), 232 (60), 187 (30), 161 (55), 159 (47), 145 (40), 131 (41), 120 (53), 119 (42), 109 (34), 107 (43), 105 (69), 95 (31), 93 (42), 91 (76), 81 (35), 79 (50), 77 (53), 69 (32), 55 (67), 53 (35), 43 (65), 41 (100), 39 (44); ^{1}H-NMR: δ 7.12 (dq, $^3J_{11,5}$= 9.7 Hz, $^4J_{11,13}$= 1.3 Hz, 1H, H-11), 4.17 (dt, $^3J_{1,2}$= 7.0 Hz, $^3J_{1,2}$= 2.3 Hz, $^3J_{1,9}$= 2.3 Hz, 1H, H-1), 3.54 (dd, $^3J_{5,11}$= 9.5 Hz, $^4J_{5,6}$= 4.9 Hz, 1H, H-5), 2.76 (m, 1H, H-9), 2.57 (dd, $^2J_{2,2}$= 17.1 Hz, $^3J_{2,1}$= 7.0 Hz, 1H, H-2), 2.23 (m, 1H, H-8), 2.18 (d, $^2J_{2,2}$= 17.1 Hz, 1H, H-2), 1.88 (m, 2H, H-7), 1.87 (d, $^4J_{13,11}$= 1.2 Hz, 3H, H-13), 1.75 (m, 2H, H-6), 1.66 (d, $^4J_{10,2}$= 0.9 Hz, 3H, H-10), 1.44 (m, 2H, H-7), 1.41 (m, 2H, H-6), 0.73 (d, $^3J_{15,8}$= 6.7 Hz, 3H, H-15); ^{13}C-NMR: δ 172.8 (C=O, C-14), 144.8 (CH, C-11), 131.4 (C, C-4), 128.4 (C, C-3), 125.7 (C, C-12), 73.2 (CH, C-1), 57.4 (CH,



1 Valerenic acid
R = H
2 Hydroxyvalerenic acid
R = OH
3 Acetoxyvalerenic acid
R = $OCOCH_3$

C-9), 47.6 (CH_2, C-2), 34.2 (CH, C-5), 31.3 (CH, C-8), 28.4 (CH_2, C-7), 25.4 (CH_2, C-6), 13.3 (CH_3, C-10), 12.7 (CH_3, C-15), 12.0 (CH_3, C-13).

Acetoxyvalerenic acid (**3**)

$[\alpha]^{20}_D$: -36.7° (c = 1.15, EtOH); UV (EtOH) λ_{max} (log ε) 217 nm (4.184); IR ν (CCl_4, max) cm^{-1}: 3500-2400 (COOH), 1735 (C=O, acetate), 1692 (C=O, α,β-unsaturated acid), 1635 (C=C); EI-MS (direct inlet) *m/z* (rel. int. %): 292 $[M]^+$ (0), 232 (58), 159 (31), 120 (100), 105 (36), 79 (45), 43 (57); ^{1}H-NMR: δ 7.07 (d, $^3J_{11,5}$= 9.7 Hz, 1H, H-11), 4.98 (dt, $^3J_{1,2}$= 7.6 Hz, $^3J_{1,2}$= 2.2 Hz, $^3J_{1,9}$= 2.2 Hz, 1H, H-1), 3.51 (dd, $^3J_{5,11}$= 9.5 Hz, $^4J_{5,6}$= 4.7 Hz, 1H, H-5), 2.81 (m, 1H, H-9), 2.58 (dd, $^3J_{2,2}$= 17.6 Hz, $^3J_{2,1}$ = 2.2 Hz, 1H, H-2), 2.22 (m, 1H, H-8), 2.18 (d, $^3J_{2,2}$= 17.6 Hz, 1H, H-2), 1.97 (s, 3H, Ac), 1.84 (s, 3H, H-13), 1.80 (m, 2H, H-7), 1.71 (m, 2H, H-6), 1.61 (d, $^4J_{10,2}$= 0.8 Hz, 3H, H-10), 1.38 (m, 2H, H-7), 1.36 (m, 2H, H-6), 0.74 (d, $^3J_{15,8}$= 6.9 Hz, 3H, H-15); ^{13}C-NMR: δ 173.4 (C=O), 171.1 (C=O), 144.6 (CH, C-11), 131.4 (C, C-4), 128.3 (C, C-3), 125.7 (C, C-12), 75.5 (CH, C-1), 54.4 (CH, C-9), 44.3 (CH_2, C-2), 34.1 (CH, C-5), 30.9 (CH, C-8), 28.2 (CH_2, C-7), 25.2 (CH_2, C-6), 21.1 (CH_3, Ac), 13.0 (CH_3, C-10), 12.5 (CH_3, C-15) 11.8 (CH_3, C-13).

Valerenal (**14**)

IR ν (neat, max) cm^{-1}: 2702 (aldehyde), 1695 and 1685 (C=O, α,β-unsaturated aldehyde), 1638 (C=C), 832 (trisubstituted C=C); EI-GC-MS *m/z* (rel. int. %): 218 $[M]^+$ (35), 203 (60), 189 (32), 185 (71), 175 (96), 161 (57), 147 (63), 145 (30), 133 (44), 121 (33), 119 (38), 107 (55), 105 (74), 95 (31), 93 (50), 91 (100), 81 (37), 79 (68), 77 (57), 55 (53), 53 (31), 43 (30), 41 (79), 39 (40); ^{1}H-NMR: δ 9.37 (s, 1H, H-14), 6.73 (dq, J = 9.4 Hz, 1H, H-6 (d), J = 1.3 Hz (q)), 3.71 (dm, J = 9.4 Hz, 1H, H-5 (d)), 2.94 (m, 1H, H-9), 1.79 (d, J = 1.3 Hz, 3H, H-13), 1.65 (m, 3H, H-10), 0.81 (d, J = 6.8 Hz, 3H, H-15); ^{13}C-NMR: δ 195.5 (CH, C-14),

155.7 (CH, C-11), 137.3 (C, C-12), 132.5, 131.8, 47.4 (CH, C-9), 37.3 (CH_2, C-2), 34.6 (CH, C-5), 32.9 (CH, C-8), 28.7, 25.3, 24.4, 13.4, 11.9, 9.1 (CH_3, C-13). The ^{1}H- and ^{13}C-NMR data were identical with the spectral data of synthetic valerenal (Baudouy *et al.*, 1983).

Valerenol (**15**)

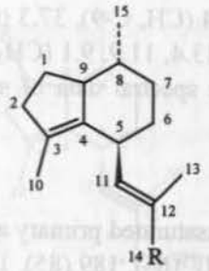
IR ν (neat, max) cm^{-1}: 3320 (OH), 1008 (C-O, α,β-unsaturated primary alcohol); EI-GC-MS *m/z* (rel. int. %): 221 $[M+1]^+$ (11), 220 $[M]^+$ (65), 189 (85), 187 (44), 147 (57), 145 (48), 133 (45), 131 (33), 121 (41), 119 (61), 107 (91), 105 (93), 95 (57), 93 (62), 91 (100), 81 (57), 79 (65), 77 (53), 55 (66), 53 (30), 43 (63), 41 (78); ^{1}H-NMR: δ 5.73 (d, J = 9.4 Hz, 1H, H-11), 3.99 (s, 2H, H-14), 3.4 (br d, J = 9.4 Hz, 1H, H-5), 2.9 (br s, 1H, H-9), 2.19 (t, J = 7.2 Hz, 2H), 1.72 (d, J = 0.7 Hz, 3H, H-13), 1.63 (d, J = 0.7 Hz, 3H, H-15), 0.76 (d, J = 6.8 Hz, 3H, H-10); ^{13}C-NMR: δ 135.2 (C, C-12), 133.1, 129.1, 127.6 (CH, C-11), 69.2 (CH_2, C-14), 47.4 (CH, C-9), 37.5 (CH_2, C-2), 33.4 (CH, C-5), 33.2 (CH, C-8), 28.7, 26.2, 24.6, 13.7 (CH_3, C-13), 13.4, 12.1.

Z-Valerenyl acetate (**16b**)

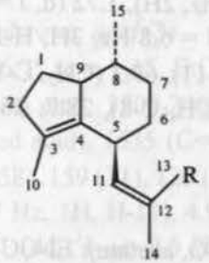
IR ν (neat, max) cm^{-1}: 1740 (C=O, acetate), 1235 (C-O, acetate): EI-GC-MS *m/z* (rel. int. %): 262 $[M]^+$ (0), 202 (43), 187 (88), 160 (51), 147 (31), 145 (65), 131 (43), 119 (33), 107 (52), 105 (58), 91 (51), 79 (31), 55 (36), 43 (100), 41 (37), 39 (34); NICI-MS: 261 $[M-1]^-$ (58), 201 (21), 59 (100); ^{1}H-NMR: δ 5.72 (br d, J ~ 9 Hz, 1H, H-11), 4.64 (s, 2H, H-13), 3.48 (br d, J ~ 9 Hz), 2.9 (br s, 1H), 2.06 (s, 3H, Me-acetate), 1.75 (d, J = 1.2 Hz, 3H, H-14), 1.62 (dt, J = 1.8 Hz (d), J = 1.0 Hz, (t), 3H), 0.75 (d, J = 6.8 Hz, 3H); trace of the *E*-isomer, with a signal at δ 4.4 (2H, H-14); ^{13}C-NMR: no off-resonance spectrum was run as only a small sample was available; in the methyl region there is a possible impurity; the intensity of the carbonyl signal was too low to be observed: δ 134.9, 132.3, 129.6, 128.4, 63.2, 47.4, 37.6, 33.4, 29.7, 29.4, 28.7, 27.0, 24.7, 21.7, 21.4, 20.9, 13.2, 12.1.

E-Valerenyl isovalerate (**17a**)

IR ν (neat, max) cm^{-1}: 1740 (C=O, ester), 1183 (C-O, ester); EI-GC-MS *m/z* (rel. int. %): 304 $[M]^+$ (0), 202 (59), 187 (100), 160 (52), 145 (54), 131 (37), 105 (40), 91 (34), 57 (57), 41 (52); NICI-MS: 303 $[M-1]^-$ (51), 201 (26), 101 (100); ^{1}H-



14 Valerenal
R = CHO
15 Valerenol
R = CH_2OH
16a *E*-Valerenyl acetate
R = CH_2OCOCH_3
17a *E*-Valerenyl isovalerate
R = $CH_2OCOCH_2CH(CH_3)_2$
18 Valerenyl valerate
R = $CH_2OCO(CH_2)_3CH_3$
19 Valerenyl hexanoate
R = $CH_2OCOC_5H_{11}$



16b *Z*-Valerenyl acetate
R = CH_2OCOCH_3
17b *Z*-Valerenyl isovalerate
R = $CH_2OCOCH_2CH(CH_3)_2$

NMR: δ 5.76 (dq, J = 9.1 Hz (d), J = 1.3 Hz (q), 1H, H-11), 4.46 (s, 2H, H-14), 3.40 (br d, 1H), 2.84 (br s, 1H), 1.70 (d, J = 1.3 Hz, 3H, H-13), 1.63 (d, J = 0.7 Hz, 3H), 0.96 (d, J = 6.6 Hz, 6H), 0.76 (d, J = 6.8 Hz, 3H); ^{13}C-NMR: δ 172.6 (C, C-1'), 135.0 (C, C-12), 131.0 (CH, C-11), 129.3, 128.6, 70.0 (CH_2, C-14), 47.5 (CH, C-9), 43.6 (CH_2, C-2'), 37.5 (CH_2, C-2), 33.5 (CH, C-5/C-8), 33.4 (CH, C-8/C-5), 28.8, 26.2, 25.8 (CH, C-3'), 24.7, 22.4 (CH_3, C-4', C-5'), 13.9 (CH_3, C-13), 13.3 (CH_3, C-10), 12.1 (CH_3, C-15); in the mixture of *E*- and *Z*-valerenyl isovalerate additional signals for *Z*-valerenyl isovalerate (**17b**) at δ 4.64 (s, 2H, H-13; ^{1}H-NMR) and 63.0 (CH_2, C-13; ^{13}C-NMR) for the -CH_2O- group.

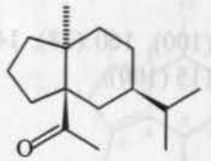
Valerenyl valerate (**18**; two isomers)
EI-GC-MS *m/z* (rel. int. %): 304 $[M]^+$ (0), 202 (81), 187 (38), 161 (71), 160 (48), 147 (31), 145 (88), 123 (62), 119 (43), 108 (38), 107 (39), 105 (96), 95 (38), 93 (48), 91 (65), 85 (58), 79 (43), 57 (100), 55 (45), 43 (32), 41 (91); NICI-MS: 303 $[M-1]^-$ (41), 201 (15), 101 (100).

Valerenyl hexanoate (**19**; two isomers)
EI-GC-MS *m/z* (rel. int. %): 318 $[M]^+$ (0), 202 (69), 187 (100), 160 (38), 145 (48), 43 (48), 41 (36); NICI-MS: 317 $[M-1]^-$ (35), 201 (55), 115 (100).

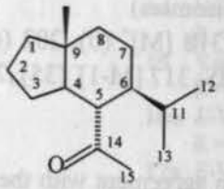
Faurinone (**22**)
^{1}H-NMR and MS data were in agreement with the data reported by Hikino *et al.* (1968). IR ν (neat, max) cm^{-1}: 1710 (C=O), 1454, 1366, 1353, 1295, 1289, 1268, 1231, 1191, 1172, 1154, 1083, 1052, 1030, 984, 958, 918, 895, 878, 862, 843, 821, 795, 772, 581, 469; ^{13}C-NMR: δ 211.8 (C, C-14), 50.9 (CH, C-4), 49.3 (CH, C-5), 47.3 (CH, C-6), 41.6 (C, C-9), 36.6 (CH_3, C-10), 32.4, 30.8 , 29.2 (CH_3, C-15), 29.0 (CH, C-11), 26.5, 23.0 (CH_3, C-13), 22.2 (CH_3, C-12), 21.4.

Faurinol-1 (one isomer of structure **22b**)
IR ν (CCl_4, max) cm^{-1}: 3625 and 3500 (OH), 1470, 1460, 1385, 1370, 1310, 1240, 1168, 1129, 1115, 1089, 1037, 1024, 996, 964, 939, 911, 893, 877, 618; EI-GC-MS *m/z* (rel. int. %): 224 $[M]^+$ (0), 206 (35), 163 (67), 123 (100), 109 (35), 95 (45), 81 (35); ^{1}H-NMR: δ 4.06 (br q, J = 6.4 Hz, 1H, H-14), 1.17 (d, J = 6.3 Hz, 3H, H-15), 1.04 (s, 3H, H-10), 0.99 (d, J = 6.1 Hz, 3H, H-12), 0.87 (d, J = 6.1 Hz, 3H, H-13); ^{13}C-NMR: δ 68.0 (C, C-14), 52.4 (CH, C-6), 46.8 (CH, C-4), 42.2 (C, C-9), 40.3 (CH, C-5), 37.7, 34.0, 33.5 (CH_3, C-10), 29.1 (CH, C-11), 27.3, 23.7 (CH_3, C-13), 22.7 (CH_3, C-12), 22.6, 21.8 (CH_3, C-15), 20.6.

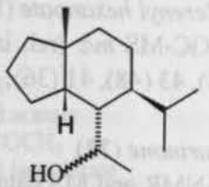
Faurinol-2 (the other isomer of structure **22b**)
IR ν (CCl_4, max) cm^{-1}: 3625 and 3460 (OH), 1470, 1460, 1378, 1247, 1170, 1108, 1089, 1066, 1053, 1027, 960, 942, 910, 891, 873, 636, 608; EI-GC-MS *m/z* (rel. int. %): 224 $[M]^+$(0), 163 (60), 123 (100), 95 (34); ^{1}H-NMR: δ 4.13 (dq, J = 6.1 Hz, J = 2.8 Hz, 1H, H-14), 1.13 (d, J = 6.3 Hz, 3H, H-15), 1.02 (s, 3H, H-10), 0.88 (d, J = 5.8 Hz, 3H, H-12), 0.87 (d, J = 5.8 Hz, 3H, H-13); ^{13}C-NMR: δ 69.0 (C, C-14), 52.4 (CH, C-6), 47.7 (CH, C-4), 42.3 (C, C-9), 41.4 (CH, C-5), 37.8, 34.3, 33.6 (CH_3, C-10), 29.2 (CH, C-11), 27.4, 23.5 (CH_3, C-12/C-13), 22.7, 20.3, 17.8 (CH_3, C-15).



22a



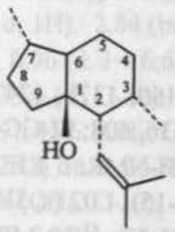
22 Faurinone



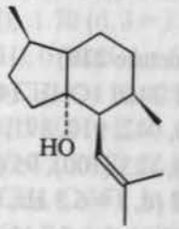
22b Faurinol

(-)-Pacifigorgiol (**28**)

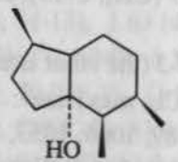
^{1}H-NMR: δ 5.06 (m, J =10.7 Hz (d), J = 1.3 Hz (m)), 2.53 (dd, J = 4.3 Hz), 1.73 (d, J = 1.3 Hz, 3H), 1.60 (d, J = 1.3 Hz, 3H), 0.96 (d, J = 6.2 Hz, 3H), 0.75 (d, J = 6.8 Hz, 3H); ^{13}C-NMR: δ 134.4 (C, C-12), 121.0 (CH, C-11),. 83.9 (COH, C-4), 49.2 (CH), 47.7 (CH), 34.9 (CH), 34.7 (CH_2), 30.3 (CH), 30.2 (CH_2), 29.4 (CH_2), 26.3 (C), 24.0 (CH_2), 19.5 (C), 19.0 (C), 18.4 (C). There are three additional substituents, two methyl groups and a hydroxyl group, so the basic ring system is a bicyclononane system. A coupled ^{13}C-NMR spectrum indicated that one of these rings was a cyclopentane ring, with two CH_2 groups and at least one CH group. The ^{13}C-NMR chemical shifts of the bridge carbon atoms correspond with those in a *trans*-bicyclo-4.3.0-nonane system (Pouet, 1982), with a hydroxyl group in one of them.



28 (-)-Pacifigorgiol



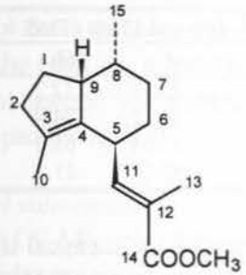
28a (+)-Pacifigorgiol



28b

Methyl valerenate (**29**)

Colourless liquid: $[\alpha]^{20}_D$: -96.6° (c = 3.08, EtOH); UV (EtOH) λ_{max} (log ε) 219 nm (4.119); IR ν_{max} (film) cm^{-1}: 2928, 2858 (OCH_3), 1714, 1642, 1435, 1379 ($COCH_3$), 1292, 1240, 1208, 1131, 1105, 1061, 840-752 (C=C); EI-GC-MS *m/z* (rel int %): 249 $[M+1]^+$ (13), 248 $[M^+]$ (74), 216 (38), 189 (32), 161 (67), 159 (36), 148 (41), 147 (44), 145 (47), 133 (94), 131 (57), 122 (51), 121 (37), 119 (56), 117 (33), 112 (37), 107 (83), 105 (100), 93 (41), 91 (65), 55 (49), 53 (30),



29 Methyl valerenate

41 (90), 39 (35); CI-GC-MS (NH_4^+): 266 $[M+18]^+$ (100), 249 $[M+1]^+$ (5); ^{1}H-NMR: δ 6.99 (dq, $^3J_{11,5}$ = 9.7 Hz, $^4J_{11,13}$ = 1.46 Hz, 1H, H-11), 3.70 (s, 3H, OCH_3), 3.51 (dd, $^3J_{5,11}$ = 9.7 Hz, $^4J_{5,6}$ = 5.0 Hz, 1H, H-5), 2.94 (m, 1H, H-9), 2.17 (bt $^3J_{2,1}$ = 7.6 Hz, 2H, H-2), 1.97 (m, 1H, H-8), 1.87 (d, $^4J_{11,13}$ = 1.46 Hz, 3H, H-13), 1.83 (m, 1H, H-7), 1.80 (m, 2H, H-1), 1.74 (m, 1H, H-6), 1.61 (m, 3H, H-10), 1.52 (m, 2H, H-1), 1.39 (m, 1H, H-6), 1.39 (m, 1H, H-7), 0.76 (d, $^3J_{15,8}$ = 7.0 Hz, 3H, H-15); ^{13}C-NMR: δ 168.9 (C=O, C-14), 143.5 (CH, C-11), 133.2 (C, C-4), 130.8 (C, C-3), 125.5 (C, C-12), 51.4 (CH_3, C-16), 47.2 (CH, C-9), 37.2 (CH_2, C-2), 34.1 (CH, C-5), 32.8 (CH, C-8), 28.5 (CH_2, C-7), 25.2 (CH_2, C-6), 24.3 (CH_2, C-1), 13.2 (CH_3, C-13), 12.1 (CH_3, C-15), 11.8 (CH_3, C-10).

Discussion

Valerenyl esters

The valerenyl esters were identified as a pair of acetates and a pair of (iso) valerates by negative ion chemical ionization (NICI) mass spectrometry (Smit and Fields, 1977; Bruins, 1979). The NICI and EI mass spectra were almost identical within each pair. Significant differences, however, were found in their ^{1}H- and ^{13}C-NMR spectra. In particular, the signals of the isobutenyl part differed in the same way as in known spectra of *E*- and *Z*-2-substituted unsaturated alcohols and their acetates (Bohlmann and Lonitz, 1980; Brunke *et al.*, 1980; Snowden *et al.*, 1981). In accordance with the combined spectral data we found valerenol, *E*- and *Z*-valerenyl acetate, *E*- and *Z*-valerenyl isovalerate, valerenyl

Table 1. ^{1}H-NMR (99.55 MHz) data for the compounds **15**, **16a/b** and **17a/b** (TMS = 0)

E	**15**	**16a**	**17a**	*Z*	**16b**	**17b**
=CH-11	5.73		5.76	=CH-11	5.72	
Me-13	1.72		1.70	Me-14	1.75	
CH_2-14	3.99	4.4	4.46	CH_2-13	4.64	4.64

Table 2. ^{13}C-NMR (25.0 MHz) data for the compounds **15**, **16b** and **17a/b** (TMS = 0)

E	**15**	**17a**	*Z*	**16b**	**17b**
C-11	127.6	131.0	C-11	132.2	132.3
C-12	135.2	135.0	C-12	134.9	
C-13	13.7	13.9	C-14	63.2	63.0
C-14	69.2	70.0	C-13	21.4	

valerate and valerenyl hexanoate. Characteristic values for the chemical shifts in the ^{1}H- and ^{13}C-NMR spectra of the *E*- and *Z*-isomers are given in Tables 1 and 2. By NICI-MS analysis of the fractions 26-34, we identified two minor components as (*Z* or *E*)-valerenyl valerate and (*Z* or *E*)-valerenyl hexanoate, in which the hexanoic acid part may be either linear or branched. During the NICI-GC-MS experiment we observed ferulic acid and bornyl acetate, known components of valerian, while three previously unknown constituents were tentatively identified, as citronellyl isovalerate (**44**), citronellyl hexanoate and methyl citronellate.

Faurinone

The results of selective ^{13}C-NMR decoupling experiments and other ^{1}H-NMR decoupling experiments with different Eu(fod)$_3$ concentrations led us to the revised structure **22** for faurinone, i.e. the configuration with an equatorial acetyl and an equatorial isopropyl group. The position of the acetyl group in structure **22a** mainly was based on the fact that only three protons were exchanged by deuteration under alkaline conditions (2N NaOD in D_2O; Hikino *et al.*, 1968), but the exchange of protons of the nucleus, dependent on the configuration, can be much hindered or take place only under very severe conditions (Thomas and Willhalm, 1965). Reduction of faurinone with lithium aluminium hydride gave two isomeric alcohols, of which the NMR spectra are in agreement with structure **22b**. The alcohols could not be detected in *V. officinalis*.

Pacifigorgiol

From the chemical shifts on addition of increasing amounts of Eu(fod)$_3$ together with decoupling experiments, the most probable structure for this alcohol was **28**. A similar compound, (+)-pacifigorgiol (**28a**) was found in a Pacific gorgonian coral *Pacifigorgia adamsii* (Izac *et al.*, 1982), the structure of which was confirmed by ^{1}H-NMR (220 MHz) and X-ray analysis of the oxidation product **28b**. The published spectral data, IR and NMR, were identical with those of the

alcohol from valerian, except for the optical rotation. (+)-Pacifigorgiol isolated from the coral has a specific optical rotation $[\alpha]^{20}_{D}$ + 41° (c = 1.02, $CHCl_3$); the alcohol isolated from valerian with an $[\alpha]^{20}_{D}$ – 45.3° (c = 0.724, $CHCl_3$) will thus be (-)-pacifigorgiol.

Methyl valerenate

Using GC-MS, we detected an unknown valerenane sesquiterpenoid with a molecular weight of 248 in the essential oil of several *V. officinalis* samples (Bos *et al.*, 1997c). The fragmentation pattern of its mass spectrum was largely similar to that of valerenic acid but it showed a clear difference of 14 *m/z* and a fragment of *m/z* 233 which corresponds with $[M-1]^+$ of valerenic acid. This could be indicative for an extra methyl group, which was readily lost. In addition, the observation that the peak of the component in the gas chomatogram did not show tailing, in contrast to valerenic acid, suggested that the methyl group was esterified to the acid function. The isolation of the unknown component was hampered by its low content, i.e. 0.1-0.2% of the essential oil. Therefore, methyl valerenate (**29**) was synthesized by treating valerenic acid with diazomethane.

Both the synthesized compound **29** and the unknown component had a retention index of 1785 in the gas chromatogram and their mass spectra were identical. Thus methyl valerenate is probably a genuine constituent of the essential oil from subterranean parts of *V. officinalis*. Methyl valerenate had already been mentioned as an intermediate in the chemical structure identification of valerenic acid (Büchi *et al.*, 1960).

In the ^{1}H-NMR spectrum of the synthetic methyl valerenate only small differences were found in comparison with the spectrum of valerenic acid (Bos *et al.*, 1986b). An additional singlet at δ 3.70 clearly indicated the presence of the esterified methyl group. The ^{13}C-NMR spectrum of valerenic acid revealed the presence of sixteen carbon atoms: eight secondary and quaternary, and eight primary and tertiary carbon signals were seen. Compared with valerenic acid, methyl valerenate had an additional tertiary carbon atom at δ 51.4 (esterified methyl) and further differences between the spectra of both compounds were small.

Chapter 3

ANALYTICAL ASPECTS OF PHYTOTHERAPEUTIC VALERIAN PREPARATIONS[3]

Introduction

The roots and rhizomes of the *Valeriana* species *V. officinalis* L. *s.l.*, *V. wallichii* DC. and *V. edulis* Nutt. ex Torr. & Gray ssp. *procera* (H.B.K.) F.G. Meyer are used for the preparation of phytomedicines employed as mild sedatives (Bos *et al.*, 1992). It is known from the literature that *V. officinalis* mainly contains valerenic acid and derivatives thereof (0.05-0.9%) as well as valepotriates (0.8-1.7%) (Hänsel and Schulz, 1982; Bos *et al.*, 1997a), whereas the two other species mainly contain valepotriates (1.8-3.5% in *V. wallichii* and 8-12% in *V. edulis*) (Wagner, 1980). Therefore, the composition of the corresponding phytomedicines largely differs (Steinegger and Hänsel, 1992). All three species are used for the production of solid, oral dosage forms (film-coated tablets and capsules), while from *V. officinalis* tinctures and a tea are also prepared. For quality assurance, standardization of such phytomedicines with respect to one or more major compounds is necessary. In addition, mutagenic effects have been described for decomposition products of the valepotriates, i.e. the baldrinals (Bounthanh *et al.*, 1981; 1983; Von der Hude *et al.*, 1985; 1986; Dieckmann, 1988; Hänsel, 1990; 1992; Keochanthala-Bounthanh *et al.*, 1993) and the absence of mutagenic constituents in valerian preparations should be proven. Thus a reliable analytical procedure is required by which the major compounds, as well as the potentially hazardous products, can be assayed (Bos *et al.*, 1994).

Several methods have been described for the analysis of the valepotriates and valerenic acid and its derivatives. An overview is given in chapter 1.

In the present study we investigated several of the HPLC methods and optimized one procedure in order to obtain a sensitive on-line HPLC method in which valerenic acid and its derivatives, as well as the valepotriates and the baldrinals could be detected in both crude plant material and phytomedicines in a

[3]This chapter is based on:
Bos R, Woerdenbag HJ, Hendriks H, Zwaving JH, De Smet PAGM, Tittel G, Wikström HV, Scheffer JJC (1996) Analytical aspects of phytotherapeutic valerian preparations. *Phytochem. Anal.* **7**, 143-151.

single run. Using this procedure, we analysed different phytotherapeutic preparations (coated tablets, capsules, tinctures and a tea) and we studied the stability of the major valerian compounds in tinctures and in two solutions with different ethanol:water ratios.

Experimental

Plant material

Subterranean parts of *V. officinalis* were obtained from the Verenigde Nederlandse Kruidencoöperatie ua, Elburg, The Netherlands.

Reference compounds

Valerenic, hydroxyvalerenic and acetoxyvalerenic acids were isolated from *V. officinalis* according to Stoll and Seebeck (1957) as described in chapter 2. Valtrate, isovaltrate, acevaltrate and didrovaltrate were isolated from *V. edulis* and purified as described earlier (Hazelhoff, 1984; Hazelhoff *et al.*, 1979b). Baldrinal and homobaldrinal were prepared from valtrate and isovaltrate, respectively (Chapter 8). Isovaleroxyhydroxydidrovaltrate (IVHD) was obtained from LAT GmbH Dr. Tittel (Munich). The identity and purity of the reference compounds were checked by melting point, IR, ^{1}H-NMR and ^{13}C-NMR spectroscopy, HPLC with diode array detection (DAD), and by mass spectrometry with positive and negative chemical ionization. As reference standards, linear calibration curves were obtained for valerenic acid, acetoxyvalerenic acid, valtrate and isovaltrate in the concentration range of 0.05-0.3 mg/ml, for didrovaltrate in the concentration range of 0.01-0.3 mg/ml, and for baldrinal and homobaldrinal in the concentration range of 0.01-0.14 mg/ml. All solutions of reference compounds were prepared in ethanol.

Ultrasonic extraction

Ground plant material (10.0 g, passing through 1 mm mesh sieve) was extracted with three portions of 30 ml methanol for 5 min in an ultrasonic bath (Bransonic 220, Branson Europe BV, Soest, The Netherlands). The extracts were filtered into a volumetric flask and the volume was adjusted to 100.0 ml with methanol.

Percolation with different ethanol-water mixtures

For each test, 10 g of ground plant material were macerated for 1 h and then percolated with ethanol-water mixtures with ratios (on a v/v basis) of 0:100, 10:90, 20:80, 30:70, 40:60, 50:50, 60:40, 70:30, 80:20, 90:10 or 100:0. The percolate (100 ml) was collected at a rate of 1 drop/2 s.

Phytomedicines

Several valerian preparations, such as tinctures, capsules and coated tablets, available in The Netherlands, were subjected to analysis. Tinctures were analysed as such; each capsule was dissolved in 100 ml methanol, and each coated tablet in 5 ml methanol. In order to prepare a tea, 3 or 5 g ground plant material were extracted with 150 ml boiling water (Wichtl, 1989; Anonymous, 1990). All samples were filtered through a 45 µm DynaGard HPLC filter (Microgon Inc., Laguna Hills, CA, USA) before injection into the HPLC apparatus.

Stability study

The stability of valtrate and isovaltrate, and of their respective decomposition products, baldrinal and homobaldrinal, was examined in: (i) a freshly prepared valerian tincture (prepared according to DAB 10; Anonymous, 1993b); (ii) a valerian tincture devoid of valepotriates and baldrinals to which 1.10 mg/ml of a mixture of valtrate and isovaltrate was added; (iii) a solution of 1.10 mg/ml of a mixture of valtrate and isovaltrate in ethanol; (iv) a solution of 1.00 mg/ml of a mixture of valtrate and isovaltrate in 70% (v/v) ethanol. The freshly prepared tincture and the solutions were stored at -20°C (control), 4°C, 20°C and 36°C. Samples were taken every day during one month and subsequently subjected to HPLC analysis.

High-performance liquid chromatography

HPLC was performed using an Isco HPLC pump 2350, an Isco gradient mixer 2360, an Isco V^{4} absorbance detector (ISCO Inc., Lincoln, NE, USA), a Kontron autosampler 360, a Kontron PC Integration pack (Kontron Instruments SpA, Milan, Italy), and a Shimadzu SPDM6A-Diode Array Detector (Shimadzu Europe GmbH, Duisburg, Germany). The chromatographic conditions were: analytical column, Superspher 100 RP-18 (5 µm; LiChrocart 250-4); guard column, LiChrospher 100 RP-18 (5 µm; LiChrocart 4-4) (Merck, Darmstadt,

Germany); eluent A, 800 g water + 156.4 g acetonitrile; eluent B, 200 g water + 625.6 g acetonitrile (both eluents contained 1 mM phosphoric acid); elution programme, first isocratic at 55% A and 45% B for 5 min, then a linear gradient to 100% B in 19 min, followed by 100% B for 2 min, subsequently a linear gradient to 55% A and 45% B in 2 min, and finally again 55% A and 45% B for 5 min; flow rate, 1.5 ml/min; start pressure, 22.5 MPa decreasing to 14.5 MPa; injected volume, 20 µl; DAD wave length, 200-600 nm; band width, 2 nm; spectrum absolute scale (mAbs), –10-500; normalization threshold, 10 mAbs.

Results and discussion

HPLC analysis

Figure 1 shows a characteristic HPLC trace of all reference compounds tested, i.e. valerenic acid and two derivatives, three diene valepotriates, two monoene valepotriates, and two baldrinals. As can be seen, all compounds are satisfac

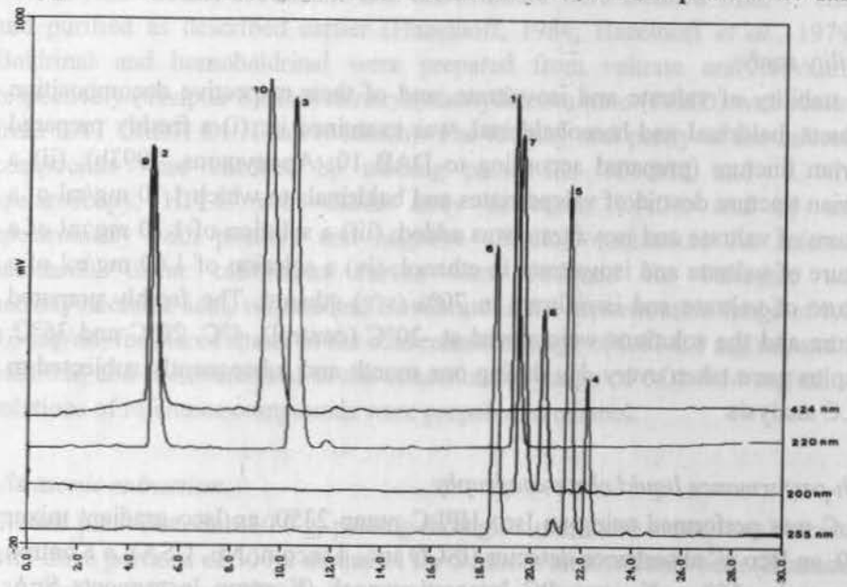


Figure 1. HPLC trace of a solution containing the reference compounds valerenic acid (**1**), hydroxyvalerenic acid (**2**), acetoxyvalerenic acid (**3**), valtrate (**4**), isovaltrate (**5**), acevaltrate (**6**), didrovaltrate (**7**), isovaleroxyhydroxydidrovaltrate (**8**), baldrinal (**9**) and homobaldrinal (**10**)

torily separated, except for valerenic acid and didrovaltrate, and for hydroxyvalerenic acid and baldrinal. These four components can, however, be identified by their respective UV maxima. In Table 1, the reference valerian compounds, separated with the HPLC system, are listed with their respective retention time, capacity factor and UV maximum.

In Figure 2 the UV spectra of the reference compounds are given; a total of four different UV maxima were found: 220-221 nm for valerenic acid and its derivatives; 255 nm for valtrate, isovaltrate and acevaltrate; 200 nm for didrovaltrate and IVHD; 424 nm for baldrinal and homobaldrinal. This means that the applied HPLC-DAD method is suitable for the on-line analysis of the three classes of valerian constituents in a single run, which can be regarded as an improvement as compared with previously published analytical procedures, that all required more than one run.

The detection limits of the different components were ca 0.01 mg/ml. The relative standard deviation found for 30 analyses was 0.4 % for valerenic acid and hydroxyvalerenic acid, 0.3 % for acetoxyvalerenic acid, 0.5 % for valtrate, 0.4 % for isovaltrate, 0.3 % for acevaltrate, 0.6 % for didrovaltrate, 0.7 % for IVHD, and 0.4 % for baldrinal and homobaldrinal. Compared with earlier

Table 1. Reference valerian compounds separated using the on-line HPLC system, with their respective retention time, capacity factor and UV maximum

Compound	Retention time (min)	Capacity factor (k')[1]	UV maximum (nm)
Baldrinal	5.00	1.99	424
Hydroxyvalerenic acid	5.39	2.21	220-221
Homobaldrinal	11.14	5.60	424
Acetoxyvalerenic acid	11.79	6.02	220-221
Acevaltrate	19.22	10.44	255
Valerenic acid	20.28	11.07	220-221
Didrovaltrate	20.31	11.09	200
Isovaleroxyhydroxy-didrovaltrate	21.16	11.60	200
Isovaltrate	22.14	12.18	255
Valtrate	22.92	12.64	255

[1]The capacity factor (k') was calculated using the formula $k' = T_R-T_0 / T_0$; T_R = retention time of compound (min) and T_0 = retention time of uracil (void time).

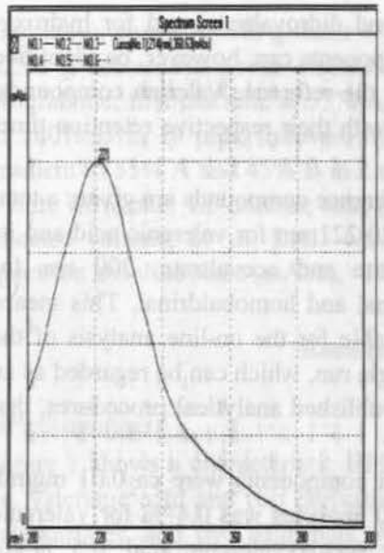
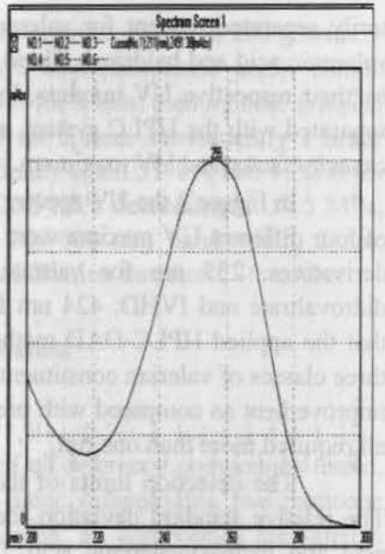


Figure 2a. UV-VIS spectrum of valerenic acid and its derivatives (**1-3**)



UV-VIS spectrum of the valepotriates (**4-6**)

work (Freytag, 1983), the sensitivity and reproducibility were markedly improved. This analytical procedure is suitable for standardization of valerian phytomedicines based on their characteristic compounds.

Since the baldrinals are mutagenic and carcinogenic compounds, it is important that these decomposition products are analysed. This is of particular importance for crude drugs and for preparations of which no information is available as to their origin. In the analytical procedures described in the literature so far (Hänsel and Schulz, 1982; Freytag, 1983), only valepotriates and valerenic acid derivatives were assayed, whilst the baldrinals have not been taken into account. Thus, the method described here is also an improvement in this respect.

Extraction of valerian constituents from V. officinalis

In order to optimize the extraction of the various secondary metabolites in question from crude plant material, we compared several solvents and extraction procedures. For valerenic acid and its derivatives, ultrasonic extraction with methanol or methylene chloride for 5 min resulted in exhaustion of the plant material. In contrast, ultrasonic extraction for 5 min with acetonitrile, ethyl acetate, ethanol and ethanol-water 70:30 did not yield a complete extraction of

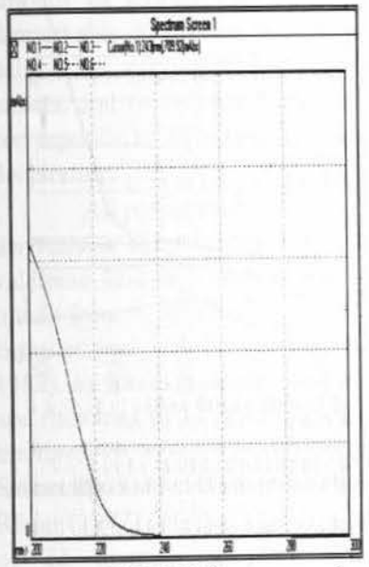
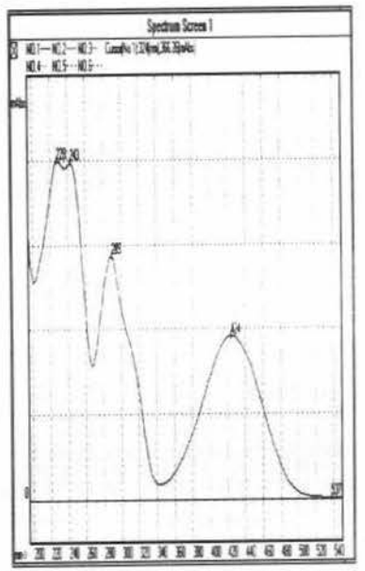


Figure 2b UV-VIS spectrum of didrovaltrate (**7**) and IVHD (**8**)



UV-VIS spectrum of baldrinal (**9**) and homobaldrinal (**10**)

valepotriates and valerenic acid and its derivatives in that period. Good results for the valepotriates were obtained via Soxhlet extraction (3 h) with ethanol.

The cultivation and selection of *V. officinalis* is based on the essential oil content and on the amount of valerenic acid and its derivatives; the species contains only relatively low levels of valepotriates (0.5-1.3%) and valerenic acid and its derivatives (0.05-0.9%). Because as many as 250-500 samples may have to be analysed in one season for cultivation trials, a fast extraction procedure is almost a prerequisite. Ultrasonic extraction (5 min) with methanol is therefore preferred for this species. In addition, unlike extracts with methylene chloride, methanolic extracts can be injected into the HPLC apparatus as such. In contrast, for the valepotriate-rich species *V. wallichii* and *V. edulis*, a Soxhlet extraction (3 h) with ethanol is needed.

In Figure 3, the influence of different ethanol:water ratios on the composition of extracts of *V. officinalis* is shown. At an ethanol concentration of 30%, valerenic acid and its derivatives started to be extracted, and at ethanol concentrations above 50% the amounts of valerenic acid, hydroxyvalerenic acid

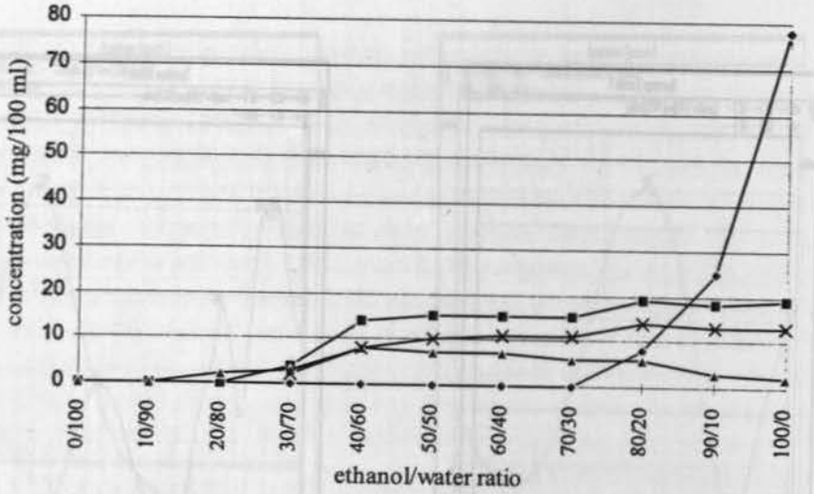


Figure 3. Extraction of **1** (■), **2** (▲), **3** (✖), and **4** and **5** (◆) from *Valeriana officinalis* with different ethanol-water mixtures

and acetoxyvalerenic acid were more or less constant. Valepotriates were extracted only at ethanol concentrations above 70%. This means that if 70% ethanol is used to prepare a valerian tincture, the extraction of the valepotriates from the (low valepotriate-containing) plant material is far from complete. In view of the potential health risk from the decomposition products of the valepotriates, a standard method should be preferred over other, mostly poorly described, isolation and processing methods.

Analysis of valerian based phytomedicines

The analytical procedure described above was also applied to a number of valerian based phytomedicines available on the Dutch market (Table 2). The phytomedicines included valerian tinctures, film-coated tablets, capsules, and preparations containing also one or more other plant extracts; in addition, some homoeopathic preparations were analysed. All tinctures had been prepared from *V. officinalis*, according to several pharmacopoeias. Film-coated tablets had been prepared from *V. officinalis* and *V. wallichii*, whereas *V. officinalis* and *V. edulis* ssp. *procera* extracts had been used for the preparation of capsules. Apart from this variation in crude drugs used to prepare the phytomedicines, we found discrepancies with respect to standardization and to recommended dosage. In

most cases, only capsules were standardized with respect to valepotriates, whilst tinctures and film-coated tablets were often not standardized; however, then the amount of extract and in several cases the plant species were declared. Remarkable differences were found with respect to the recommended maximum daily dose, varying from 45 to 180 drops (2-8 ml), from 6 to 12 film-coated tablets, and from 3 (27.5 mg/capsule) to 8 capsules (48 mg/capsule); the latter corresponds to 82.5-384 mg valepotriates per day based on the manufacturer's declaration.

All preparations were analysed for their contents of valerenic acid and its derivatives, valepotriates, and baldrinals: the results are listed in Table 2. Only valerenic acid and acetoxyvalerenic acid were found in the tinctures, in a tea (made from *V. officinalis),* in several of the film-coated tablets, and in one of the capsules analysed. This is in agreement with an earlier study (Hänsel and Schulz, 1982), in which hydroxyvalerenic acid was not detected in 50 samples of roots and rhizomes of *V. officinalis*, the crude material for the preparation of valerian tinctures. However, hydroxyvalerenic acid has been detected by others (Stoll and Seebeck, 1957; Hänsel and Schulz, 1985; Closson *et al.*, 1992). Schimmer and Röder (1992) detected valerenic acid and acetoxyvalerenic acid by TLC in 19 out of 23 commercial plant drugs, but all three compounds (valerenic, hydroxyvalerenic and acetoxyvalerenic acids) have been detected in several extracts by Hänsel and Schulz (1985). We propose that hydroxyvalerenic acid is found as a decomposition product of acetoxyvalerenic acid when the crude drug is stored at a too high humidity.

Neither valepotriates nor baldrinals were detected in the tinctures, as could be expected, or in the film-coated tablets containing valerian extracts. Despite the presence of valepotriatés in Valmane®, no baldrinals were found: this may be explained by the finding that more than 80% of the valepotriates were present as didrovaltrate, and decomposition of this compound does not appear to yield baldrinals (Van Meer *et al.*, 1977).

The fact that we did not detect valepotriates in most of the phyto-therapeutic preparations analysed, is in agreement with previous studies concerning crude root material as well as phytomedicines. Our tests further showed that baldrinals were only present in capsules containing high initial concentrations of valepotriates. Schild (1969) found valepotriates in freshly prepared tinctures, but they were no longer detectable after 4 weeks. Laufer *et al.* (1970), Van Meer *et al.* (1977), and Hänsel and Schulz (1985) did not detect valepotriates in commercially available *Extractum Valerianae* as well as in other

Table 2a. Valerian phytomedicines (tinctures and tea) available on the Dutch market with their (acetoxy)valerenic acid content, analysed with the on-line HPLC system described

Product name[1]	Manufacturer/ Importer	Declaration of manufacturer	VAD[2] (mg/100 ml)	Daily dose (max)[3] dr
Tinctura Valerianae	Genfarma	Ph. Ned. VI	25.1	n.i.[4]
Valeriaan D0	Biohorma	HAB 1	6.0	150 dr[7]
Valeriaan druppels	Dara Pharma	Infusum Valerianae Conc. B.P.C. '63	n.d.[5]	180 dr
Valeriaan tinctuur	Tendo-Haco	Ned. Pharm. VI	4.4	60 dr
Valeriaan Sch 28	Pflüger	n.i.	n.d.	45 dr
Valeriana D0	VSM	HAB 1	16.2	n.i.
Valeriana D6	VSM	HAB 1	n.d.	n.i.
Valeriana complex	Bonusan	n.i.	2.8	60 dr
Valeriana officinalis	Bonusan	n.i.	8.8	n .i.
Valeriana Similiaplex	Pascoe	n.i.	1.0	45 dr
Valera-H	Lumen Naturae	n.i.[6]	n.d.	90 dr
Valeriana D0 (1984-1990)	Biohorma	HAB 1	6.2-20.8	n.i.
Valeriana D0 (1982-1990)	VSM	HAB 1	1.9-13.9	n.i.
Valerian Tea		3-5 g	3.6-5.3	10.8-15.9 mg/150 ml

[1]The ownership of all registered product names and trademarks of manufacturers of products in this list is acknowledged; [2]VAD (valerenic acid + acetoxyvalerenic acid); [3]recommended by manufacturer; [4]not indicated; [5]not detected (< 1 mg/ml); [6]complex; [7]dr = drops.

Table 2b.Valerian phytomedicines (film-coated tablets, capsules) available on the Dutch market with their respective (acetoxy)valerenic acid, valepotriate and baldrinal content analysed with the on-line HPLC system described

Product name[1]	Manufacturer/ Importer	Declaration of manufacturer	VAD[2] (mg)	VPTR[7] (mg)	Baldr[8] (mg)	Daily dose (max)[3]
Calmolan	NL-Pharma	0.5 mg valerenic acid	0.55	n.d.[5]	n.d.	3 tablets
Extr. Valerianae 45	Pharma Chemie	45 mg extractum Valerianae spissum	0.09	n.d.	n.d.	6 tablets
Extr. Valerianae spis.	Pharma Chemie	idem	0.09	n.d.	n.d.	n.i.[4]
Valeriaan extact 45	Pharbita	idem	0.11	n.d.	n.d.	6 tablets
Valeriaan extract	Pharbita	idem	0.09	n.d.	n.d.	6 tablets
Valdispert	Kali Chemie	45 mg valerian extract	0.05	n.d.	n.d.	9 tablets
Valmane	Kali Chemie	50 mg VPTR[7]	n.d.	50.2	n.d.	6 tablets
Rode Valeriaan	Bional	140 mg extract radix Valerianae	0.10	n.d.	n.d.	n.i.
Valeriana complex	Bonusan	*V. officinalis*[6]	n.d.	n.d.	n.d.	12 tablets
Valeriana *capsules*	VSM	n.i.	n.d.	n.d.	n.d.	n.i
Valeriaan	Arkopharma	270 mg radix *V. officinalis*	2.0	n.d.	n.d.	6 capsules
Bional Nachtrust	Bional	100 mg extract radix Valerianae	n.d.	n.d.	n.d.	2 capsules
Bioform	Bioform	27.5 mg valepotriates	n.d.	10.6	0.03	82.5 mg VPTR
Nervex capsules	Roter	50 mg valepotriates (*V. edulis*)	n.d.	46.6	0.07	300 mg VPTR
Sanox capsules	Chefaro	48 mg valepotriates	n.d.	51.6	0.08	384 mg VPTR
Natuvit capsules	Medicosma	27.5 mg valepotriates	n.d.	34.8	n.d	82.5 mg VPTR
Valeriaan	Beiersdorf	extract Valerianae[6]	n.d.	8.3	n.d.	3 capsules

[1]The ownership of all registered product names and trademarks of manufacturers of products in this list is acknowledged; [2]VAD (valerenic acid + acetoxyvalerenic acid); [3]recommended by manufacturer; [4]not indicated; [5]not detected (< 1 mg/tablet or capsule); [6]complex; [7]VPTR (valtrate + isovaltrate + acevaltrate); [8]Baldr (baldrinal + homobaldrinal).

phytomedicines. Braun *et al.* (1986) detected baldrinals in 14 out of 52 phytomedicines available in Germany using TLC and HPLC.

Stability of valerian constituents

In order to study the stability of the valepotriates and to gain an insight into the formation of their decomposition products baldrinal and homobaldrinal, samples of a freshly prepared valerian tincture from *V. officinalis* were stored at 4°C, 20°C and 36°C, and subsequently analysed. Figure 4 shows that the valepotriates could no longer be detected after a storage period of 2 weeks at 36°C. After a storage period of 1-2 weeks at 20°C, only about 36% of the original level of the valepotriates was still present, whilst the tincture stored at 4°C still contained 67% of the original level of the valepotriates after 4 weeks. Despite the rapid decomposition of the valepotriates at higher temperatures, the presence of baldrinals could not be detected using the HPLC-DAD.

In order to investigate to what extent valepotriates are converted into baldrinals, a solution of 1.10 mg/ml of valtrate and isovaltrate in ethanol, a solution of 1.00 mg/ml of valtrate and isovaltrate in 70% ethanol, as well as valepotriate-free and baldrinal-free valerian tinctures to which a mixture consisting of valtrate and isovaltrate had been added, were stored at different temperatures. In the ethanol solution, decomposition of the valepotriates did not

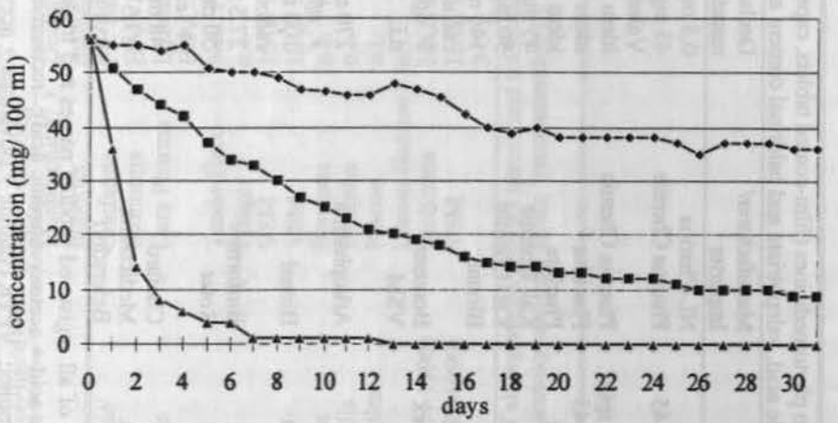


Figure 4. Decomposition of valtrate and isovaltrate in a freshly prepared tincture from *Valeriana officinalis* stored at 4 °C (◆), 20 °C (■), and 36 °C (▲)

occur, and baldrinals were not found: this solution remained colourless. In contrast, in 70% ethanol, the concentration of valepotriates decreased to 30% of the original level after 2 weeks at 20°C, and to almost nothing after 3-4 weeks, while the baldrinal content rapidly increased from 5% (after 2 weeks) to 85% (after 3-4 weeks). This solution rapidly became yellow. The amount of baldrinals was lower then might be expected from the valepotriate content, and probably other artefacts were formed.

During storage at 36°C, the valepotriate content of the tincture also rapidly decreased (Figure 4), but apparently baldrinals were hardly found, as mentioned above. A part of the baldrinals may react with other, yet unknown, constituents present in a tincture, and this may explain the lack of baldrinals in stored tinctures.

According to some literature, the quality of *Tinctura Valerianae* may be related to the presence of valepotriates or of valerenic acid and its derivatives. However, valepotriates showed a temperature-dependent instability and could not be detected after a relatively short storage period (Figure 5). Their decomposition products baldrinal and homobaldrinal, that were found in the

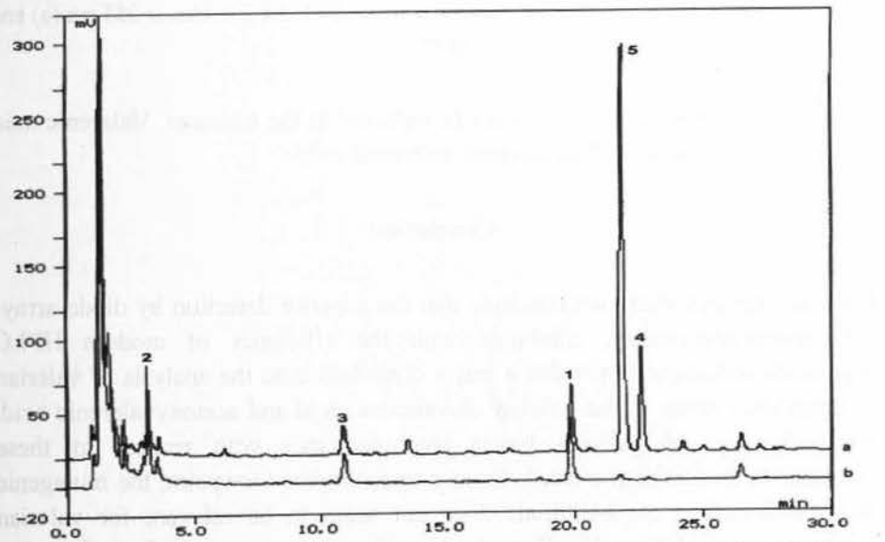


Figure 5a. HPLC trace of a freshly prepared valerian tincture, at 255 nm (a) and 220 nm (b); numbers of peaks are as in Figure 1

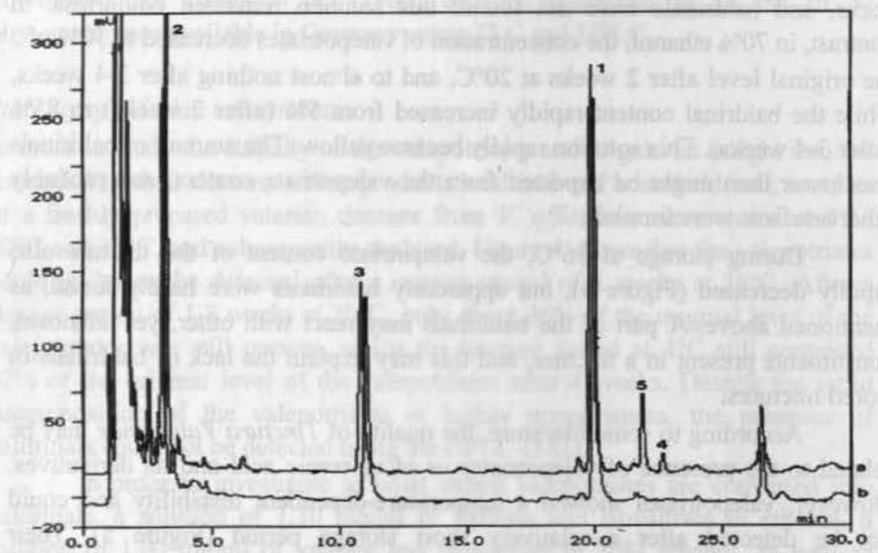


Figure 5b. HPLC trace of a valerian tincture stored at 20°C for 2 weeks, at 255 nm (a) and 220 nm (b); numbers of peaks are as in Figure 1

ethanolic [70%] solutions, could not be detected in the tinctures. Valerenic acid and acetoxyvalerenic acid, in contrast, remained stable.

Conclusion

From the present study we conclude that the superior detection by diode array-UV spectrophotometry, combined with the efficiency of modern HPLC separation techniques, provides a major contribution to the analysis of valerian compounds. Owing to the stability of valerenic acid and acetoxyvalerenic acid, standardization of valerian based phytomedicines with respect to these compounds should be preferred. From a toxicological viewpoint, the mutagenic activity described for baldrinals does not seem to be relevant for valerian tinctures prepared from *V. officinalis* as well as for a tea made from the same species. It is, however, not known if other decomposition products, that may be formed, possess such activity. Water is a prerequisite for the decomposition of valepotriates into baldrinals in ethanolic solutions.

Phytomedicines are complex mixtures of substances, and the crude plant material varies largely in its content of the active ingredients. Additional alterations of active ingredients may be induced by the manufacturing process. Therefore the demands of the manufacturer can only be met by comprehensive quality control. This control should include the crude plant material, the manufacturing process as well as standardization of the final product (Bauer *et al.*, 1994). We recommend with respect to valerian based phytomedicines that not only the identity of the plant material and the extraction procedure should be provided, but also the amount of the components present in the preparations by which they may be standardized.

For the sake of uniformity, it is therefore desirable that the note for guidance as published in *The Rules Governing Medicinal Products in the European Community* (Anonymous, 1975a) will be implemented in all countries where phytomedicines are available on the market.

Chapter 4

THE ESSENTIAL OIL FROM, AND VALERENIC ACID AND VALEPOTRIATES IN SUBTERRANEAN PARTS OF *VALERIANA OFFICINALIS* L. *S.L.* AND SEVERAL CLOSELY RELATED TAXA[4]

Introduction

The roots and rhizomes of *Valeriana officinalis* L. *s.l.*, family Valerianaceae, are used for the preparation of phytomedicines that are employed as mild sedatives (Bos *et al.*, 1994; 1996). The plant, that is included in the European Pharmacopoeia, is cultivated as a medicinal plant on a commercial scale in the northern part of Europe. *V. officinalis* is a collective term; hence the addition *s.l.* (*sensu lato*). Within the collective species several subspecies are distinghuished, that differ from each other cytologically, and by their degree of ploidy, their morphology and their area of distribution (Bos *et al.*, 1994; 1996; 1997a). In a number of morphologically and karyologically defined types of *V. officinalis*, differences in valepotriate contents and composition as well as in the contents of characteristic essential oil compounds have been found (Titz *et al.*, 1982; 1983).

In the literature some confusion seems to exist regarding the subspecies that are part of the collective species. The following four subspecies are generally listed in textbooks: *V. officinalis* ssp. *officinalis* L.; *V. officinalis* ssp. *collina* (Wallr.) Nyman; *V. officinalis* ssp. *sambucifolia* (Mikan f.) Celak; and *V. repens* Host (synonym *V. procurrens* Wallr.) (Frohne and Jensen, 1992; Steinegger and Hänsel, 1992; Bruneton, 1995). Additionally, *V. exaltata* Mikan and *V. pratensis* Steud. are mentioned (Steinegger and Hänsel, 1992). The subspecies *V. officinalis* ssp. *officinalis* is diploid ($2n = 14$); it is a perennial herb with a hollow and grooved stem bearing a rosette of leaves at the base, and opposite pinnatisect leaves on the stem. The leaves comprise 11-19 lanceolate folioles, all of the same width. The subspecies is common in damp

[4]This chapter is based on:
Bos R, Woerdenbag HJ, Hendriks H, Scheffer JJC (1997) Composition of the essential oils from underground parts of *Valeriana officinalis* L. *s.l.* and several closely related taxa. *Flavour Fragr. J.* **12**, 359-370.
Bos R, Woerdenbag HJ, Hendriks H, Scheffer JJC (1997) Occurrence of valerenic acid and valepotriates in taxa related to *Valeriana officinalis* L. *s.l. Sci. Pharm.* **65**, 165-168.

woods and meadows, as well as on dry, elevated grounds in Europe and in the temperate zones of Asia (Hegnauer, 1973). The three other generally listed subspecies have similar morphological characteristics (Weberling, 1970). *V. officinalis* ssp. *collina* (Wallr.) Nyman ($2n$ = 28) has leaves with 15-27 folioles, all of the same width, and *V. officinalis* ssp. *sambucifolia* (Mikan f.) Celak ($2n$ = 56; synonym *V. excelsa* Poir.) has leaves with 5-9 folioles, with the apical one clearly larger than the others. *V. repens* Host ($2n$ = 56) is the fourth subspecies (Bruneton, 1995). Often intermediates occur which are even common in certain regions; it is therefore not certain how some of the described taxa should be accommodated (Weberling, 1970; Ockendon 1976).

The aim of the present study was to investigate the essential oils isolated from the subterranean parts of the different subspecies in order to get insight into similarities and differences as to their composition. In order to find out whether further similarities or differences exist in the secondary metabolites pattern of these species, the plant material was also analysed for valerenic acid and valepotriates. For the same reason we investigated the essential oil content and composition as well as the valerenic acid and valepotriate contents of several taxa that are closely related to *V. officinalis* L. *s.l.*

Experimental

Plant material

Seeds of four different subspecies of *V. officinalis* L. *s.l.*, i.e. *V. officinalis* ssp. *officinalis*, V. *officinalis* ssp. *collina* (Wallr.) Nyman, *V. officinalis* ssp. *sambucifolia* (Mikan f.) Celak and *V. repens* Host, and of the related taxa investigated, i.e. *V. angustifolia* Tausch, *V. salina* Pleijel, *V. tuberosa* L., *V. montana* L., *V. rossica* P. Smirnov, *V. phu* L., *V. pyrenaica* L. and *V. tripteris* L., were obtained from several botanical gardens in Europe (Table 1). The samples 1 and 38 were already available as dried subterranean material at the Department of Pharmaceutical Biology, Groningen; samples 3-7 existed of plant material for commercial purposes, the samples 15 and 16 were collected in Ramsau am Dachstein (Styre, Austria) at an altitude of 1100 m, and sample 27 was obtained as dried subterranean plant material from Prof. Dr Teppner (Graz, Austria). The seeds, except for the samples 3-7, were germinated at the botanical garden of the University of Groningen. Subsequently, the small plants were transferred to an experimental field in Elburg. The soil consisted

of sand, pH 5.6. The planting distance was 50 x 30 cm. Voucher specimens have been deposited at the Department of Pharmaceutical Biology, Groningen.

Isolation procedure

Each essential oil sample was isolated from 20.0 g of air-dried and freshly ground (1 mm) root material by hydrodistillation for 4 h in 300 ml water, according to the determination of the essential oil content in vegetable drugs, using the apparatus described in the '*Nederlandse Farmacopee*', 6th edn, 2nd printing (Anonymous, 1966b). Xylene (100 µl) was used as the collection liquid, and the oil samples were stored at -20°C until analysed. The oil samples were diluted 50 times with cyclohexane prior to GC and GC-MS analysis.

Gas chromatography

GC analysis was performed on a Hewlett-Packard 5890 Series II gas chromatograph equipped with a 7673 injector and a Hewlett Packard 3365 Series II Chemstation, under the following conditions: column, WCOT fused-silica CP-Sil 5 CB (25 m x 0.32 mm i.d., film thickness 0.25 µm; Chrompack, Middelburg, The Netherlands); oven temperature programme, 50-290°C at 4°C/min; injector temperature, 250°C; detector (FID) temperature, 300°C; carrier gas, nitrogen; inlet pressure, 5 psi; linear gas velocity, 26 cm/s; split ratio, 56:1; injected volume, 1.0 µl.

Gas chromatography-mass spectrometry

GC-MS (EI) was performed on a Finnigan 9500/3300/6110 GC-MS computerized system. The GC conditions were: column, WCOT fused-silica CP-Sil 5 CB (25 m x 0.25 mm i.d., film thickness 0.25 µm; Chrompack); oven temperature programme, 60-300°C at 6°C/min; injector temperature, 250°C; carrier gas, helium; inlet pressure, 5 psi; linear gas velocity, 30 cm/s; split ratio, 12:1. MS conditions: ionization energy, 70 eV; ion source temperature, 185°C; interface temperature, 290°C; scan speed, 1 scan/s; mass range, 34-500 u. The reactant gas mixture for negative ion chemical ionization (NICI) was CH_4-N_2O, ca 1:1 (Smit and Field, 1977; Bruins, 1983).

The identity of the components was assigned by comparison of their retention indices, relative to C_9-C_{21} *n*-alkanes, and mass spectra with corresponding data of reference compounds and from the literature (Adams, 1989; Tucker and Maciarello, 1993). The percentages of the components were calculated from the GC peak areas, using the normalization method.

Table 1. Origin, essential oil yield, and valerenic acid and valepotriate contents (calculated on a dry weight basis) of subterranean parts of various valerian (sub)species

Sample	Plant material[1]	Origin and number	Oil yield (%, v/w)	Valerenic acid (%)	Valepotriates (%)
Valeriana officinalis L. *s.l.*					
1	*V. officinalis* 'polka'	Rauscholzhausen [D]	1.55	n.d.	n.d.
2	*V. officinalis*	Unknown Botanical Garden	0.36	0.13	1.08
3	*V. officinalis*	VNK Elburg [NL]	0.69	0.25	0.45
4	*V. officinalis*	VNK Elburg (8570) [NL]	0.95	0.30	0.21
5	*V. officinalis*	VNK Elburg (8545) [NL]	0.83	0.38	0.15
6	*V. officinalis*	VNK Elburg (TS) [NL]	0.22	0.11	0.73
7	*V. officinalis*	VNK Elburg (TS) [NL]	0.26	0.08	0.88
8	*V. officinalis*	Botanical Garden Jena (1049) [D]	0.44	0.03	0.02
9	*V. officinalis*	Botanical Garden Jena (1093) [D]	0.31	0.13	0.61
10	*V. officinalis*	Alpengarten Belvedere Vienna (997/H10) [A]	0.34	0.04	0.12
11	*V. officinalis*	Botanical Garden Poznan [PL]	0.33	0.07	0.58
12	*V. officinalis*	Unknown Botanical Garden	0.65	0.12	0.14
13	*V. officinalis*	Botanical Garden Braunschweig (486) [D]	0.73	0.13	0.08
14	*V. officinalis*	Botanical Garden Göttingen [D]	1.10	n.d.	0.04
15	*V. officinalis*[2]	Ramsau am Dachstein (1988) [A]	1.18	n.d.	0.02
16	*V. officinalis*[2]	Ramsau am Dachstein (1988) [A]	1.23	n.d.	0.02
17	*V. collina* (Wallr.) Nyman	Botanical Garden Stuttgart-Hohenheim (1113) [D]	0.85	n.d.	0.05
18	*V. collina* (Wallr.) Nyman	Botanical Garden Stuttgart-Hohenheim [D]	0.78	0.21	0.05

Table 1. continued

19	*V. sambucifolia*	Botanical Garden Warsaw [PL]	1.24	n.d.	n.d.
20	*V. sambucifolia*	Botanical Garden Heidelberg [D]	1.57	n.d.	0.03
21	*V. sambucifolia* Mikan F. ssp. *sambucifolia*	Botanical Garden Turku (438/117, 1988) [FIN]	1.13	0.10	0.08
22	*V. sambucifolia* Mikan F. ssp. *sambucifolia*	Botanical Garden Turku (15,117/384, 1988) [FIN]	0.20	0.29	0.15
23	*V. sambucifolia*	Unknown Botanical Garden (216/90)	0.45	n.d.	0.59
24	*V. sambucifolia*	Botanical Garden Poznan (1944) [PL]	0.19	0.08	0.98
25	*V. sambucifolia*	Botanical Garden Budapest (147) [H]	0.21	0.02	0.73
26	*V. repens* Host	Unknown Botanical Garden (787, 147/90)	0.34	0.04	0.34
Related valerian taxa					
27	*V. celtica* ssp. *norica* Vierh.	Prof. Teppner, Graz [A]	0.20	n.d.	n.d.
28	*V. angustifolia* Tausch	Botanical Garden Warsaw [PL]	1.10	0.04	n.d.
29	*V. salina* Pleijel	Botanical Garden Leipzig [D]	0.80	0.16	0.10
30	*V. tuberosa* L.	Botanical Garden Szeged [H]	1.02	0.06	0.10
31	*V. montana* L.	Botanical Garden Jena [D]	2.20	n.d.	0.02
32	*V. montana* L.	Botanical Garden Szeged [H]	1.35	0.09	0.12
33	*V. rossica* P. Smirnov	Botanical Garden Leipzig [D]	0.90	0.05	0.10
34	*V. phu* L.	Unknown Botanical Garden (217/90)	0.41	n.d.	0.41
35	*V. phu* L.	Botanical Garden Zürich (1017/89) [CH]	0.33	n.d.	0.23
36	*V. pyrenaica* L.	Botanical Garden Zürich (1018/89) [CH]	0.35	0.21	0.58
37	*V. tripteris* L.	Botanical Garden Jena (2944) [D]	0.38	n.d.	0.73
38	*V. exaltata* Mikan	Unknown Botanical Garden (1987)	1.00	n.d.	0.73

[1]Name as received from the botanical garden; [2]collected from wild population; n.d. = not detected.

High-performance liquid chromatography

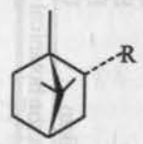
Valerenic acid and derivatives, and valepotriates were analysed by HPLC as described in chapter 3.

Results and discussion

V. officinalis L. *s.l.*

The essential oil yield for the 16 samples of *V. officinalis* ssp. *officinalis* investigated varied between 0.22% and 1.55% (v/w) calculated on a dry weight basis (Table 1, samples 1-16). The well-known component of valerian oil, bornyl acetate (**30b**, 2-36%), was present in all of these samples (Table 2). Other components that were found in all oils were nojigiku acetate (**31**, trace-0.8%), myrtenyl acetate (**32**, trace-9%), valerenic acid (0.3-3%) and a tertiary sesquiterpene alcohol with a retention index (RI) of 1597. Other components present, but not in all samples, were the monoterpene ester myrtenyl isovalerate (**32a**), the sesquiterpene hydrocarbon eudesma-2,6,8-triene (**33**), and the oxygenated sesquiterpenoids kessane, (-)-pacifigorgiol, ledol (**34**), β-eudesmol (**35**), valerianol, valeranone, valerenal, valerenol and α-kessyl acetate, as well as two unknown sesquiterpene alcohols with an RI of 1548 and 1622, respectively. In only one sample (12) a pacifigorgiol isomer (RI = 1473) was detected.

According to the mass spectra, a kessanyl acetate isomer was detected in nine samples of *V. officinalis* (1-3, 6-8, 10, 11,13), together with kessoglycyl monoacetate (**27a/b**), that was found in four of the samples (7-10). In the samples 5 and 10 faurinone was found. Based on a comparison of the mass spectra of β-eudesmol and valerianol, and because of an abundant peak at *m/z* 59, it was concluded that also other tertiary sesquiterpene alcohols were



30 Borneol
R = OH

30a Bornyl formate
R = OOCH

30b Bornyl acetate
R = $OCOCH_3$

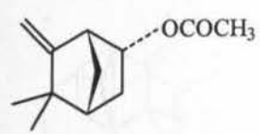
30c Bornyl isovalerate
R = $OCOCH_2CH(CH_3)_2$

30d Bornyl hexanoate
R = $OCO(CH_2)_4CH_3$

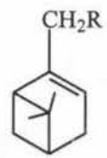
(RI = 1568, 1597, 1602, 1622, 1635). In two samples (9 and 10) the yet unidentified sesquiterpene alcohol with an RI of 1622 was the main component. An unidentified sesquiterpene acetate (RI = 1746) was present in some of the samples. We also found an unknown component (RI = 1148) with a molecular weight of 138. In an earlier report, two unidentified components with a molecular weight of 138 were detected in a hydrodistillate from *V. officinalis* flowers (Brunke *et al.*, 1992). Possibly these compounds are of a comparable nature. Methyl valerenate (RI = 1785) was, as far as we know, found for the first time to be present in the essential oil of *V. officinalis* (samples 2-11). The identity of methyl valerenate was confirmed by synthesis using valerenic acid and diazomethane (chapter 2; Bos *et al.*, 1997f). The compound had already been synthesized from valerenic acid in 1965 (Büchi *et al.*, 1960; Krepinsky *et al.*, 1965), but until now it was not reported to occur in nature.

The essential oil content of *V. officinalis* roots and rhizomes, either wild grown or cultivated, has been reported to vary from 0.1% to 2.8% (Gildemeister and Hoffmann, 1961; Evans, 1989; Steinegger and Hänsel, 1992; Morazzoni and Bombardelli, 1995). In the monograph for *Valerianae radix* in the European Pharmacopoeia an essential oil content of at least 0.5% is required. Both the essential oil content and its composition may strongly differ from plant to plant (Hendriks *et al.*, 1981b). Also seasonal variations play a role in the essential oil amount (Schaette, 1971; chapter 7).

The two samples (17 and 18) of the subspecies *collina* yielded 0.78% and 0.85% oil (v/w), which was more than the data (about 0.3%) mentioned in the literature so far (Lemberkovics *et al.*, 1977). Bornyl acetate was the main component in both samples (22% and 24% respectively) which is in accordance with previous findings (Titz *et al.*, 1983). Myrtenyl acetate, kessane, camphene (**36**) and β-eudesmol were main components in both samples. In sample 18 (-)-pacifigorgiol was present, but not in sample 17. Also



31 Nojigiku acetate

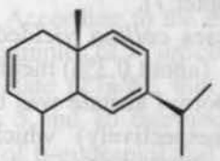


32 Myrtenyl acetate
R = $OCOCH_3$

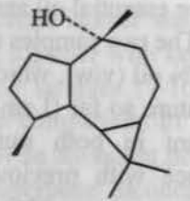
32a Myrtenyl isovalerate
R = $OCOCH_2CH(CH_3)_2$

the sesquiterpene alcohol with an RI of 1548 was only present in sample 18. The second main component in sample 17 was the sesquiterpene alcohol with an RI of 1622. The sesquiterpene acetate (RI=1768) was only present in sample 17. Another sesquiterpene acetate (RI=1867) was present in sample 18 in an amount of 14%, but amounted to only 0.2% in sample 17. According to the literature, large differences in the composition of the essential oil of *V. collina* exist (Titz *et al.*, 1983; Corsi *et al.*, 1984).

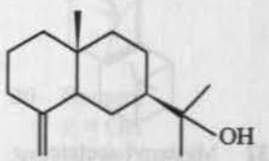
The valerian subspecies *sambucifolia* (Table 1, samples 19-25) showed a large variation in the yield (0.19-1.57%, v/w) of oil as well as in its composition, the literature also saying that large differences in the essential oil composition exist within this subspecies (Titz *et al.*, 1983). Bornyl acetate together with valerianol were the main components in all cases. In some of the samples other main components were also found, e.g. camphene (2-8%), β-eudesmol (2-8%), myrtenyl acetate (0.2-7%), kessane (0.7-8%) and valeranone (0.2-10%). Drimenol (**37**), which was already identified earlier as oil component of *V. dioica* and *V. edulis* (Becker, 1984), was found in four samples (19, 21-23). A recent investigation of *V. officinalis* var. *sambucifolia* revealed 58 identified components; the main components were bornyl acetate and valerenal (Gränicher *et al.*, 1995). The latter compound was found in



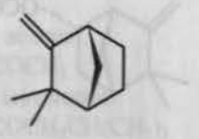
33 Eudesma-2,6,8-triene



34 Ledol



35 β-Eudesmol



36 Camphene

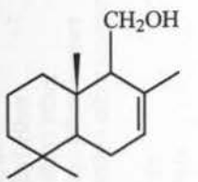
concentrations of 0.3% to 4% in the samples we analysed. We found valerianol as one of the main components (3-34%); valerenic acid was found in relatively small amounts in all samples (0.1-5%).

V. repens oil, yield 0.34% (v/w), contained bornyl acetate, kessane and valerianol as the main components. Based on the high kessane content, this subspecies seems to be chemotaxonomically closer to the valerian species occurring in Japan than to those in Europe (Nishiya *et al.*, 1992; Oshima *et al.*, 1995).

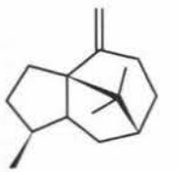
Other valerian taxa

The essential oil content of the roots of the related taxa, namely *V. celtica* ssp. *norica, V. angustifolia, V. salina, V. tuberosa, V. montana, V. rossica, V. phu, V. pyrenaica, V. tripteris and V. exaltata* (samples 27-38), varied from 0.20% to 2.20% (v/w), calculated on a dry weight basis (Table 1). Bornyl acetate was, except in the samples 34-36, the main component with percentages between 6% and 46%.

From the related *Valeriana* taxa, *V. celtica* (Table 3a, sample 27) is the best known species (Endlich, 1966; 1973). There are three subspecies, i.e. *V. celtica* ssp. *celtica* in the Alps, *V. celtica* ssp. *norica* in the eastern Alps of Austria, and *V. celtica* ssp. *pancicii* in the Montenegro area (Weberling *et al.*, 1971). *V. celtica* ssp. *norica*, investigated in this study, is native to the 'Niedere Tauern' and to the south side of the 'Hohe Tauern' at an altitude of at least 1800 m (Weberling, 1970; Weberling *et al.*, 1971; Bicchi *et al.*, 1983). This subspecies has been of economic importance in the region of Styria (Austria) for about 2000 years. Since the 15th century, the rhizomes were dug in the Alps and traded extensively in Europe for fragrance purposes (Teppner, 1982). We found that the main oil components were bornyl acetate, myrtenyl acetate and patchouli alcohol. The presence of bornyl acetate and patchouli alcohol is in agreement with earlier investigations (Bicchi *et al.*, 1983; Becker, 1984).



37 Drimenol



38 γ-Patchoulene

Table 2. Percentage composition of the essential oils isolated from roots and rhizomes of four subspecies of *V. officinalis* L. *s.l.*

Component	Retention index[a]	Sample 1-16[b]	17	18	19-25	26	M[c]	BP[d]
Tricyclene	919	tr-0.1	tr	tr	tr-0.1	tr	136	93
α-Pinene	926	0.1-2.2	0.4	0.9	0.6-2.7		136	93
Fenchene	930					0.8	136	93
Camphene	937	0.1-6.4	**4.3**	**6.9**	1.8-**8.1**	5.0	136	93
Sabinene	960	tr-0.1	tr		tr-0.9	0.1	136	93
β-Pinene	964	tr-0.9	0.3	0.7	0.2-1.2	0.6	136	93
β-Myrcene	980	tr-0.1			tr		136	43
Unknown	989	tr-0.2				0.2	144	101
α-Phellandrene	993				tr		136	93
p-Cymene	1008	0.1-1.5	0.1	0.4	0.1-1.1	0.7	134	119
1,8-Cineole	1015	tr-0.2				tr	154	43
β-Phellandrene	1016	tr-2.4	0.3	0.5	0.1-1.7	0.2	136	93
Limonene	1017	0.1-1.2			0.1-0.5	0.3	136	68
γ-Terpinene	1047	tr-0.2	tr		tr-0.2	0.1	136	93
Terpinolene	1075	tr-0.1			tr	tr	136	93
Linalol	1082	tr	tr		tr - 0.2	tr	154	71
Menthenal isomer	1098	tr-0.3	0.1		0.1-0.3	0.1	152	94
Menthenal isomer	1110	tr-0.1	0.1		tr-0.2		152	94
Camphor	1112	tr-0.1				tr	152	95
Isoborneol	1132	tr-0.1					154	95
Borneol	1140	0.1-0.6	0.4	0.7	0.2-3.0	0.7	154	95
$C_9H_{14}O$	1148	tr-0.1					138	96
Terpinen-4-ol	1154	0.1-0.6	0.1	0.3	0.1-0.9	0.2	154	71
α-Terpineol	1165	tr-0.1	tr	tr	tr-0.2	tr	152	59
Myrtenol	1171	0.1-4.0	0.6	0.2	0.1-1.3	0.6	152	79
Unknown	1173	0.1					166	135
cis-Carveol	1174	0.1-0.2			0.2-0.8		152	84

Table 2. (continued)

Unknown	1183	tr-0.1					166	135
trans-Carveol	1191	tr-0.1	tr		tr-0.3	tr	152	84
Cumin aldehyde	1203	tr-0.1					148	133
Thymol methyl ether	1207	tr-0.4	tr	0.3	tr-0.6	tr	164	149
Carvacrol methyl ether	1211	0.1-0.2	0.1	0.1	0.1-0.2	0.4	164	149
Thymol methyl ether isomer	1216	0.1-0.2	0.1	0.1	tr-0.2	0.2	164	149
Carvacrol methyl ether isomer	1222	tr-0.2	tr	tr			164	149
Geraniol	1233				tr		154	69
Linalyl acetate	1239				0.1		196	93
Bornyl acetate	1264	2.3-**35.5**	**23.5**	**22.2**	**4.3-25.1**	**12.7**	196	95
$C_{12}H_{20}O_2$	1268	tr-0.1					196	121
Nojigiku acetate	1272	tr-0.8	0.6	0.6	0.1-1.6	0.3	194	107
Unknown	1286	tr-0.2					162	162
Thymol	1290	0.1					150	135
Myrtenyl acetate	1299	tr-**9.1**	**5.8**	**7.9**	0.2-**7.0**	3.9	194	91
Eugenol	1305	tr-0.1					164	164
δ-Elemene	1326	0.2-2.9	1.3	0.4	0.2-4.4	0.3	204	121
Citronellyl acetate	1332	tr-0.1	tr		tr		198	81
$C_{15}H_{24}$	1340	0.1-0.3		0.5	tr-0.3	0.1	204	93
α-Ylangene	1357	0.1-0.4		0.7	tr-0.4	0.1	204	105
α-Copaene	1366	tr-0.2	0.1	0.1	tr-0.2	0.1	204	105
2,5-Dimethoxy-*p*-cymene	1372				tr-0.1		194	179
$C_{15}H_{24}$	1374	tr-1.6	tr	0.1	tr-0.2	0.1	204	147
β-Elemene	1379	0.1-0.4	0.3	0.3	0.1-0.7	0.1	204	81
$C_{15}H_{24}$	1388				0.1	0.1	204	94
$C_{15}H_{24}$	1394					0.4	204	93
$C_{15}H_{24}$	1395	0.1-1.9	0.7	1.0			204	82
2,6-Dimethoxy-*p*-cymene	1396	0.2-1.4	0.5	0.3	0.2-0.9	0.1	194	179
$C_{15}H_{24}$	1396	0.3-1.3			0.5-2.3		204	105
$C_{15}H_{24}$	1400	tr-0.9	0.1	0.2	0.2-0.3		204	147

Table 2. (continued)

Component	Retention index[a]	Sample 1-16[b]	17	18	19-25	26	M[c]	BP[d]
$C_{15}H_{24}$	1403	0.1-2.4	0.7		0.6-2.5		204	41
$C_{15}H_{24}$	1404					1.4	204	93
$C_{15}H_{24}$	1407	tr-0.1					204	105
$C_{15}H_{24}$	1418	0.1-4.7	0.1		0.1-1.6	0.1	204	161
γ-Patchoulene	1423				0.1-0.5		204	122
$C_{15}H_{24}$	1424				0.1-0.2		204	81
$C_{15}H_{24}$	1425	0.1-0.5	0.1	0.3	0.1-0.4	0.2	204	93
$C_{15}H_{22}$	1432	tr-0.1			0.1-0.2		202	91
β-Bisabolene	1439	0.2-0.8					204	69
α-Humulene	1440	0.1-0.9	0.7	0.5	0.1-3.6	0.6	204	93
$C_{15}H_{24}$	1442	tr-2.1					204	41
Eudesma-2,6,8-triene	1443	0.5-**7.6**	0.5	1.7	0.3-**5.9**	1.4	204	105
$C_{15}H_{24}$	1450	0.1-0.2	0.1		0.1-**6.5**	0.1	204	93
β-Ionone	1454	0.1-0.7	0.3		0.1-0.3		192	177
$C_{15}H_{24}$	1454	0.1-0.4					204	43
$C_{15}H_{24}$	1457				0.3-0.6		204	121
$C_{15}H_{24}$	1457					0.2	204	81
$C_{15}H_{24}$	1462	1.6				0.5	204	105
ar-Curcumene	1464	0.7-2.1	1.4	0.8	0.1-2.1	0.7	202	119
$C_{15}H_{24}$	1464	1.2					204	93
Germacrene-D	1465	0.2-1.4	0.6				204	161
$C_{15}H_{24}$	1467	0.1-1.2					204	93
α-Guaiene	1469				0.1-3.1		204	93
$C_{15}H_{24}$	1472	0.1-0.5	0.8			0.7	204	161
Pacifigorgiol isomer	1473	1.0					222	110
$C_{15}H_{24}$	1476				0.5-4.1		204	41
$C_{15}H_{24}$	1479	0.1-**4.2**	1.0		1.0-1.9	1.3	204	93
γ-Elemene	1482	0.1-0.4			0.1-0.2		204	81
$C_{15}H_{24}$	1486	0.1-0.6					204	93

Table 2. (continued)

$C_{15}H_{24}$	1486		0.3		0.3-0.9		204	119
$C_{15}H_{24}$	1488	0.2					204	133
$C_{15}H_{22}$	1488	0.1					202	159
$C_{15}H_{24}$	1489				0.3-1.1		204	93
α-Bulnesene	1489					0.4	204	107
$C_{15}H_{24}$	1492	0.2-0.6	0.2	0.6		0.2	204	119
Bornyl isovalerate	1492	0.5-1.2	0.9	0.4	tr-0.1	0.2	238	95
$C_{15}H_{26}O$	1494	0.6-0.8			0.5-1.2	0.7	222	69
$C_{15}H_{24}$	1495					0.2	204	93
β-Gurjunene	1498	tr-1.0	1.3		0.4-4.1	0.9	204	122
Kessane	1507	0.2-3.8	**6.3**	3.3	0.7-**8.2**	**7.8**	222	43
δ-Cadinene	1507	0.1-0.3			0.1-1.1		204	161
$C_{15}H_{24}$	1517				0.1-0.2		204	93
$C_{15}H_{26}O$	1522	**4.1-5.4**	0.4				222	59
Pacifigorgiol	1523	0.5-**3.2**		**3.0**	0.4-**2.5**	0.9	222	110
Faurinone	1526	0.4-0.6			0.3-0.9		222	43
$C_{15}H_{24}$	1527	0.3-0.8	0.4	0.5		0.5	204	93
Myrtenyl isovalerate	1535	1.6-**10.5**	1.4	1.8	0.5-2.7	3.3	236	91
$C_{15}H_{24}$	1535	0.8-**4.5**					204	121
Maaliol	1541	0.1-1.6			0.2-1.2		222	43
$C_{15}H_{24}O$	1548	0.9-**6.1**		**4.9**	0.2-2.8	0.8	220	43
$C_{15}H_{24}O$	1552	0.4-1.3				2.3	220	41
$C_{15}H_{26}O$	1555	0.2-4.2	0.8	1.4	0.2-0.7		222	121
Citronellyl isovalerate	1558	0.2-2.1	0.1	0.4	0.2-1.4	0.4	240	81
$C_{15}H_{24}O$	1561	0.1-0.6	0.2				220	43
$C_{15}H_{24}O$	1565				0.2-1.1		220	69
$C_{15}H_{26}O$	1565	0.4-0.9		0.6			222	69
$C_{15}H_{26}O$	1568	0.6-2.7	1.1			1.3	222	59
Ledol	1575	0.5-2.1	0.8	1.0	0.3-1.5	1.4	222	43

Table 2. (continued)

Component	Retention index[a]	Sample 1-16[b]	17	18	19-25	26	M[c]	BP[d]
$C_{15}H_{24}O$	1578	0.1-0.5					220	93
$C_{15}H_{26}O$	1591				0.3-1.2		222	161
$C_{15}H_{26}O$	1592	0.2-1.6	0.8			0.6	222	43
$C_{15}H_{24}O$	1597				**2.2-7.5**	**4.5**	220	119
$C_{15}H_{24}O$	1597	1.4-**13.8**					220	59
$C_{15}H_{24}O$	1597		3.0	2.0			220	161
$C_{15}H_{26}O$	1602	0.2-**9.7**				0.7	222	161
$C_{15}H_{26}O$	1602				0.4-**5.1**		222	121
Unknown	1605	0.3 -**10.9**	0.6				252	41
γ-Eudesmol	1605				0.5-0.7		222	161
β-Eudesmol	1613	0.6-**6.9**	3.9	**5.1**	1.9-**8.3**	**5.5**	222	59
$C_{15}H_{26}O$	1622	**14.5-15.3**	**19.5**				222	59
$C_{16}H_{28}O_2$ (isovalerate)	1623	0.3-0.4					252	57
Valerianol	1625	1.5-**14.2**		1.1	2.6-**33.9**	**19.0**	222	161
$C_{15}H_{26}O$	1626	0.2-2.1		0.9			222	43
$C_{15}H_{26}O$	1635	0.9-2.3					222	59
Valeranone	1639	0.5-**8.2**	0.8	0.5	0.2-**10.4**	1.1	222	98
Myrtenyl hexanoate	1640	0.1-0.2		0.5			250	91
Cryptofauronol	1644	0.4-3.9			0.1-2.7		238	41
$C_{15}H_{26}O$	1645	0.5-1.0	0.6	0.4	tr-0.9	0.6	222	82
$C_{15}H_{26}O$	1653	0.1-1.2					222	43
epi-α-Bisabolol	1659	0.5-1.5	0.7		0.5-1.9	0.6	222	69
$C_{17}H_{28}O_2$ (isovalerate)	1677	0.3-0.9	0.2			0.5	264	57
Valerenal	1688	0.4-**12.4**	0.5	1.6	0.3-**3.8**	0.6	218	91
$C_{15}H_{24}O$	1692					0.1	220	43
$C_{16}H_{30}O_2$ (isovalerate)	1693	0.1-0.3	0.1		0.3-3.8		254	57
Valerenol (*cis* or *trans*)	1698	0.3-1.2	0.2		0.4-1.1	0.4	220	91

Table 2. (continued)

Eugenyl isovalerate	1728	0.1-0.4			0.1-1.9	0.3	248	164
$C_{17}H_{28}O_2$ (acetate)	1746	1.3-**7.1**				0.5	264	75
Drimenol	1750	0.1-0.4			0.1-**4.4**		222	109
$C_{17}H_{26}O_2$ (acetate)	1768	0.4-2.6			0.3-0.8		262	43
$C_{17}H_{28}O_2$ (acetate)	1768		**2.2**			0.3	264	43
α-Kessyl acetate	1772	0.1 -**12.6**			0.3-2.1		280	43
cis-Valerenyl acetate	1777	0.2-0.8		0.4	0.2-0.6	0.3	262	43
Methyl valerenate	1785	0.1-0.2					248	91
trans-Valerenyl acetate	1796	0.1-1.5			0.1-0.7		262	43
Isoeugenyl isovalerate	1814	0.1-1.1	0.3		0.1-0.9		248	164
Kessanyl acetate	1827	0.1-**3.5**	0.2	1.4			280	43
Valerenic acid	1831	0.3-2.8	0.2	0.2	0.1-**4.7**	0.1	234	91
An isovalerate	1850	tr-0.3						57
$C_{17}H_{28}O_2$ (isovalerate)	1855	0.2-0.6					264	57
$C_{17}H_{26}O_2$ (acetate)	1867	tr-**10.2**	0.2	**13.8**	tr-2.9	0.1	262	43
$C_{17}H_{32}O_3$ (isovalerate)	1881	tr-1.1			0.1-1.5		284	57
Cryptofauronyl acetate	1890	tr-2.9	tr		tr-0.9	0.1	280	43
Methyl palmitate	1908	0.1-0.9	0.1				270	74
Eugenyl hexanoate	1917				tr-**4.9**		262	164
Palmitic acid	1946	0.1-1.9	0.2		0.1-0.3	0.3	256	60
Kessoglycyl monoacetate	1972	0.1-0.7					296	43
cis-Valerenyl isovalerate	1984	tr-0.7		0.2	tr-0.1	0.1	304	57
Ethyl palmitate	1986	tr-0.6					284	88
$C_{20}H_{34}O_2$ (isovalerate)	2004	0.1-1.1	0.1				306	57
trans-Valerenyl isovalerate	2024	tr-1.6	tr		tr-1.7	0.4	304	57
$C_{20}H_{32}O_2$ (isovalerate)	2051	tr-0.7					304	57

[a]Retention Index relative to C_9-C_{21} *n*-alkanes on the CP-Sil 5 column; [b]tr = trace (<0.05%);[c]M = molecular weight; [d]BP = base peak (100%).

Also *V. phu* (Table 3b, samples 34 and 35) is already known for about 2000 years; it was used for medicinal purposes in the 16th century in the southern part of Europe (Weberling, 1970). So far little was known about the chemical composition of the essential oil. In the samples we analysed, bornyl acetate was only present in small amounts, whereas a sesquiterpene hydrocarbon (RI = 1418), γ-patchoulene (**38**), patchouli alcohol, a valerenal isomer (RI = 1701) and patchoulyl acetate (**20a**), together with some unknown oxygen-containing sesquiterpenes (RI = 1548, 1564, 1565, 1592, 1674) were the main components. Patchouli alcohol has been reported only once as a constituent of *V. officinalis* (Rücker and Tautges, 1976), but it can not be excluded that the investigated commercially obtained dry roots and rhizomes in that study were from *V. phu* instead of *V. officinalis*. In view of the presence of patchouli alcohol, *V. phu* probably belongs to the subclasses to which also *V. wallichii* and *V. fauriei* belong (Nishiya *et al.*, 1995).

In *V. exaltata* (sample 38) the main components were bornyl acetate, valeranone, valerenal, a sesquiterpene hydrocarbon (RI = 1445) and two sesquiterpene alcohols (RIs = 1548 and 1659). Earlier investigations on *V. exaltata* showed large differences in the composition of its oil. So far, bornyl acetate, an unknown sesquiterpene, valeranone and valerenal had been found (Titz *et al.*, 1983). The published mass spectrum of that unidentified sesquiterpene shows a great similarity to the spectrum of bornyl isovalerate (**30c**) recorded in our study, while the published mass spectra of two unidentified sesquiterpene alcohols (Titz *et al.*, 1983) are identical with those of β-eudesmol and valerianol.

Data on the essential oil composition of *V. angustifolia, V. salina, V. tuberosa, V. montana, V. rossica, V. pyrenaica* and *V. tripteris* could not be found in the literature (Table 3). Within these taxa, the largest amount of bornyl acetate (46%) was detected in the oil of *V. angustifolia* (sample 28), and the smallest amount in that of *V. pyrenaica* (sample 36). Valerianol was present in all of these samples. The largest amount of valerianol (20%) was found in the oil of *V. pyrenaica* and the smallest amount was found in one of the oils of *V. montana*. Main unidentified sesquiterpene hydrocarbons were found in *V. montana* (sample 32; RI = 1443), in *V. tuberosa* (sample 30; RI =1445) and in *V. rossica* (sample 33; RI = 1445). Furthermore, a number of unidentified oxygen-containing sesquiterpenes were found in several samples.

Valerenic acid could be detected in most of the essential oils, but the amounts were smaller than the total content in extracts from the subterranean plant material as analysed by HPLC.

The results of the HPLC analysis are also given in Table 1. Valerenic acid and its derivatives have been recognized as lead compounds of *V. officinalis* L. *s.l.*, and are considered characteristic of this collective species (Steinegger and Hänsel, 1992). We now show that valerenic acid is also present in other *Valeriana* taxa that, according to the textbooks, do not belong to the collective species, namely *V. angustifolia*, *V. montana*, *V. pyrenaica*, *V. rossica*, *V. salina* and *V. tuberosa*. In contrast, no valerenic acid could be detected in *V. exaltata*.

Valepotriates are known to be present in many *Valeriana* species, and are regarded as characteristic of the entire genus. Here we report the presence and content of valepotriates in several species that were not known to contain these compounds until now. In the cases that valepotriates could not be detected, this may be ascribed to decomposition as valepotriates are known to be labile when the plant material has been stored for a longer time.

Based on the results of the present study, the following conclusions may be drawn. If valerenic acid is considered to occur solely in *V. officinalis* L. *s.l.*, the following taxa should also belong to this collective species, namely *V. angustifolia, V. montana, V. pyrenaica, V. rossica, V. salina* and *V. tuberosa*, whereas *V. exaltata* should not be part of it. On the other hand, it is possible that valerenic acid is not unique for the collective species *V. officinalis* as currently defined. In that case, the other species containing valerenic acid are possibly closely related taxa.

Table 3a. Percentage composition of the essential oils isolated from roots and rhizomes of some related valerian taxa

Component	Retention index[a]	Sample 27[b]	28	29	30	31	32	33	M[c]	BP[d]
Tricyclene	919				tr	tr			136	93
α-Pinene	926	0.1	0.1	0.4	1.2	0.7	0.4	0.1	136	93
Camphene	937	0.3	0.9	**3.7**	**4.0**	**5.9**	3.0	0.2	136	93
Sabinene	960			tr	0.1	0.1	0.1	tr	136	93
β-Pinene	964	tr	0.2	0.2	0.5	0.8	0.4	tr	136	93
β-Myrcene	980					tr			136	43
p-Cymene	1008		0.1	0.2	0.2	0.2	0.2	tr	134	119
1,8-Cineole	1015						0.5	tr	154	43
β-Phellandrene	1016							0.2	136	93
Limonene	1017			0.3	0.7	0.7	0.4	tr	136	68
γ-Terpinene	1047				tr	tr	tr	tr	136	93
Terpinolene	1075				0.1	0.1	tr		136	93
Linalol	1082			tr				tr	154	71
Menthenal isomer	1098			tr	tr	tr		0.1	152	94
Camphor	1112		0.2			tr		tr	152	95
Borneol	1140	0.5	1.2	0.4	0.5	0.2	0.2	0.3	154	95
$C_9H_{14}O$	1148			tr					138	96
Terpinen-4-ol	1154			0.2	0.3	0.2	0.2	0.4	154	71
Myrtenol isomer	1160		0.2	0.2	tr	tr			152	79
α-Terpineol	1165				tr	tr		tr	152	71
Myrtenol	1171			0.2	0.5	0.3	0.3		152	79
cis-Carveol	1174							0.1	152	84
Thymol methyl ether	1207			tr	0.2	tr	0.1	0.3	164	149
Carvacrol methyl ether	1211			0.2	0.2	0.3	0.1	0.2	164	149
Thymol methyl ether isomer	1216			0.1	0.1	0.3	0.2	0.2	164	149
Carvacrol methyl ether isomer	1222			tr					164	149
Bornyl acetate	1264	**22.1**	**46.2**	**13.5**	**11.7**	**23.4**	**19.4**	**10.1**	196	95
Nojigiku acetate	1272	0.5	0.8	0.6	0.3	0.6	0.6	0.1	194	107

Table 3a. (continued)

Myrtenyl acetate	1299	**3.0**	**5.7**	**5.8**	**3.5**	**9.9**	**7.5**	0.3	194	91
δ-Elemene	1326	0.3	0.5	0.6	1.9	1.6	1.0	1.7	204	121
Citronellyl acetate	1332					0.3	0.1		198	81
$C_{15}H_{24}$	1340		0.2	0.5	0.2	0.1	0.2	0.2	204	93
α-Ylangene	1357				0.1				204	105
α-Copaene	1366				tr	0.1	tr	0.1	204	105
$C_{15}H_{24}$	1369					tr		0.1	204	105
2,5-Dimethoxy-*p*-cymene	1372		0.1			tr		0.1	194	179
$C_{15}H_{24}$	1374				0.1		0.2	0.1	204	105
β-Elemene	1379			0.2	0.3	0.4	0.2	0.5	204	81
$C_{15}H_{24}$	1388							tr	204	94
$C_{15}H_{24}$	1394				0.8	0.4	1.1		204	93
$C_{15}H_{24}$	1395			0.9	0.5				204	82
2,6-Dimethoxy-*p*-cymene	1396		0.9	0.5					194	179
$C_{15}H_{24}$	1400				0.2			1.5	204	147
$C_{15}H_{24}$	1404				1.1	1.5	1.6	2.0	204	93
α-Santalene	1409				0.1				204	94
$C_{15}H_{24}$	1418				2.6	0.1	0.2	1.3	204	161
$C_{15}H_{24}$	1418					0.1			204	121
γ-Patchoulene	1423	1.7							204	122
$C_{15}H_{24}$	1424						0.2		204	81
$C_{15}H_{24}$	1425			0.3	0.2	0.2		0.3	204	93
$C_{15}H_{24}$	1432		0.3		0.1		tr	0.1	204	69
$C_{15}H_{22}$	1433			0.2					202	91
$C_{15}H_{22}$	1439			0.3					202	91
α-Humulene	1440				0.3	0.9		0.4	204	93
$C_{15}H_{24}$	1443				0.2		**8.0**		204	105
$C_{15}H_{24}$	1444		0.8			0.3			204	69
$C_{15}H_{24}$	1445			1.4	**6.4**			**6.7**	204	105

Table 3a. (continued)

Component	Retention index[a]	27[b]	28	29	Sample 30	31	32	33	M[c]	BP[d]
$C_{15}H_{24}$	1450					0.1			204	93
$C_{15}H_{24}$	1450				0.3		0.1	0.4	204	91
β-Ionone	1454			tr	0.3	0.5	0.5	0.6	192	177
$C_{15}H_{24}$	1454			0.2					204	43
ar-Curcumene	1464	0.3	0.6	0.7	tr	2.2	1.3	tr	202	119
Germacrene-D	1465				1.8			3.0	204	161
α-Guaiene	1469							0.1	204	93
$C_{15}H_{24}$	1471		0.3	0.4					204	69
$C_{15}H_{24}$	1473			0.1	0.2	0.9			204	119
$C_{15}H_{24}$	1473			0.1				0.5	204	105
Pacifigorgiol isomer	1473				0.4		0.5		222	110
$C_{15}H_{24}$	1476			0.3		1.3			204	161
$C_{15}H_{24}$	1479				3.0			1.7	204	93
$C_{15}H_{24}$	1479					1.0	2.8		204	161
$C_{15}H_{24}$	1486				0.2	0.5	0.2	0.3	204	93
α-Bulnesene	1489	0.4	0.9	0.3	0.3		0.1	0.3	204	107
$C_{15}H_{24}$	1492		0.4				0.6		204	119
Bornyl isovalerate	1492	0.1	0.2	0.1	0.1	0.5	0.4	0.2	238	95
$C_{15}H_{26}O$	1494	0.2	0.6	0.3	0.5	1.0		0.3	222	69
$C_{15}H_{24}$	1495		0.3	0.1	0.2	0.4		0.1	204	93
β-Gurjunene	1498			0.1	0.2	1.3	0.2		204	122
$C_{15}H_{24}O$	1501		tr	0.2	0.1			0.2	220	147
Kessane	1507				1.0	0.2	0.9	0.4	222	43
$C_{15}H_{26}O$	1521							0.5	222	93
$C_{15}H_{26}O$	1522	0.7	1.3			0.8			222	59
Pacifigorgiol	1523			2.6	1.0		1.1	1.3	222	110
Faurinone	1526						0.3		222	43
$C_{15}H_{24}$	1527				0.6	0.6		0.3	204	93
Myrtenyl isovalerate	1535	1.1	2.3	**3.9**	2.7		1.6		236	91

Table 3a. (continued)

$C_{15}H_{24}$	1535					1.3		2.1	204	121
$C_{15}H_{24}O$	1541				**7.2**		0.5	2.1	220	43
Maaliol	1541					tr			222	43
$C_{15}H_{26}O$	1548	0.8	1.3	**3.9**	1.1		1.1	2.0	222	91
$C_{15}H_{24}O$	1552	1.0		2.3		0.9	1.3	0.4	220	41
$C_{15}H_{26}O$	1555		2.6		0.7				222	121
Citronellyl isovalerate	1558			2.1	0.3	0.1	0.3	0.6	240	81
$C_{15}H_{26}O$	1565			1.0	0.5			0.8	222	69
$C_{15}H_{26}O$	1568					3.0	1.6		222	59
Ledol	1575	0.6	0.6	1.7	0.7	0.6	0.9	0.9	222	43
$C_{15}H_{24}O$	1578					0.6			220	93
$C_{15}H_{24}O$	1591						0.4		220	135
$C_{15}H_{26}O$	1592			0.4	0.5	0.6		1.4	222	43
$C_{15}H_{24}O$	1597	0.3	0.5	2.1	**4.0**	3.0	2.5	**9.3**	220	119
$C_{15}H_{26}O$	1602			0.8	0.3				222	43
$C_{15}H_{22}$	1603				0.2			0.1	202	159
$C_{15}H_{24}O$	1605				0.5	0.8	0.7	0.7	220	69
β-Eudesmol	1613	0.6	0.8	**6.3**	1.0	3.2	1.6	**6.8**	222	59
$C_{15}H_{26}O$	1617	0.4	1.0						222	43
Valerianol	1625		3.0	**13.9**	3.2	**15.6**	2.8	**13.6**	222	161
Patchouli alcohol	1625	**5.4**							222	83
$C_{15}H_{26}O$	1635					0.7			222	59
Valeranone	1639	1.2	0.4		2.5		3.3	0.7	222	98
Myrtenyl hexanoate	1640			0.6					250	91
$C_{15}H_{26}O$	1645		0.5	0.5	0.5	0.6	0.5	0.8	222	82
epi-α-Bisabolol	1659	0.2	0.2	0.3	0.6	0.8	1.0	0.7	222	69
$C_{17}H_{28}O_2$ (isovalerate)	1677	0.7	1.5	1.1	0.6	0.3	0.5	0.6	264	57
Valerenal	1688		0.2	0.2	**4.5**		**8.8**	3.7	218	91
$C_{15}H_{24}O$	1692	0.4	0.8	0.2		0.1		0.2	220	43

Table 3a. (continued)

Component	Retention index[a]	Sample 27[b]	28	29	30	31	32	33	M[c]	BP[d]
Valerenol (*cis* or *trans*)	1698				0.6		1.1	0.4	220	91
Eugenyl isovalerate	1728	0.9	1.5	1.3	0.1		0.3	0.3	248	164
Drimenol	1750	1.1	2.4	0.9					222	109
$C_{17}H_{28}O_2$ (acetate)	1768				1.5	0.6	0.6	1.9	264	43
α-Kessyl acetate	1772					0.1	0.2		280	43
cis-Valerenyl acetate	1777		0.3		0.5		0.4	0.6	262	43
trans-Valerenyl acetate	1796				0.7		0.4	0.8	262	43
Isoeugenyl isovalerate	1814	0.3	0.7	2.5	1.0	0.2		1.0	248	164
Kessanyl acetate	1827	0.7		1.2	2.3	0.2	1.8		280	43
Valerenic acid	1831	0.3	0.3		1.1				234	91
$C_{17}H_{26}O_2$ (acetate)	1867	0.3	0.6	0.7	2.8		3.2	0.2	262	43
$C_{17}H_{32}O_3$ (isovalerate)	1881		0.4	0.9					284	57
Cryptofauronyl acetate	1890		0.4		0.1	0.1	0.1	0.1	280	43
Palmetic acid	1946		0.6	0.4	0.2		0.3	0.2	256	60
cis-Valerenyl isovalerate	1984				0.2	tr	0.5	0.2	304	57
trans-Valerenyl isovalerate	2024				0.7		0.5	0.4	304	57

[a]Retention Index relative to C_9-C_{21} *n*-alkanes on the CP-Sil 5 column; [b]tr = trace (<0.05%);[c]M = molecular weight; [d]BP = base peak (100%).

Table 3b. Percentage composition of the essential oils isolated from roots and rhizomes of some related valerian taxa

Component	Retention index[a]	Sample 34[b]	35	36	37	38	M[c]	BP[d]
Tricyclene	919	tr	tr		tr		136	93
α-Pinene	926	0.4	0.4	0.5	0.3	0.2	136	93
Camphene	937	1.1	0.9	0.8	**9.7**	0.7	136	93
Sabinene	960	tr	tr	tr	0.1	0.1	136	93
β-Pinene	964	0.2	0.2	0.3	1.2	0.1	136	93
β-Myrcene	980	tr	tr				136	43
Unknown	989	tr	0.2	tr	0.2		144	101
p-Cymene	1008	0.5	0.5	1.1	0.4	0.5	134	119
1,8-Cineole	1015	tr	tr	tr	tr		154	43
β-Phellandrene	1016	0.4	0.4	0.3	0.3		136	93
Limonene	1017				0.4		136	68
γ-Terpinene	1047			0.1	0.1	tr	136	93
Terpinolene	1075			0.1	tr	tr	136	93
Linalol	1082				tr	0.1	154	71
Menthenal isomer	1098				0.1		152	94
Camphor	1112				0.1		152	95
Isoborneol	1132				0.1		154	95
Borneol	1140	tr	tr	0.2	0.9	0.5	154	95
Terpinen-4-ol	1154	0.1	0.1	0.2	0.3	0.1	154	71
Myrtenol isomer	1160				tr		152	79
α-Terpineol	1165	tr	tr	tr	tr		152	71
Myrtenol	1171				1.5		152	79
cis-Carveol	1174	0.2	0.2	0.6			152	84
trans-Carveol	1191			tr	tr		152	84
Thymol methyl ether	1207			tr			164	149
Carvacrol methyl ether	1211			tr			164	149
Thymol methyl ether isomer	1216			tr			164	149
Carvacrol methyl ether isomer	1222				0.1		164	149

Table 3b. (continued)

Component	Retention index[a]	Sample 34[b]	35	36	37	38	M[c]	BP[d]
Bornyl acetate	1264	0.9	0.9	2.3	**22.5**	**5.9**	196	95
Nojigiku acetate	1272	tr	0.1	0.1	0.3	tr	194	107
Myrtenyl acetate	1299	0.1	tr	0.1	**4.9**	0.5	194	91
δ-Elemene	1326	0.1	0.1	0.5	0.3	0.8	204	121
Citronellyl acetate	1332					0.1	198	81
$C_{15}H_{24}$	1340	tr	tr	0.1	tr	0.4	204	93
α-Ylangene	1357	0.1	0.1	tr	tr		204	105
α-Copaene	1366	0.1	0.1		0.1		204	105
$C_{15}H_{24}$	1369	0.7	0.6				204	105
$C_{15}H_{24}$	1374			0.3		0.3	204	105
β-Elemene	1379	0.1	0.1	0.1	0.1	0.2	204	81
$C_{15}H_{24}$	1388					0.9	204	94
$C_{15}H_{22}$	1389	0.8	0.9				202	159
$C_{15}H_{24}$	1394			0.3			204	93
$C_{15}H_{24}$	1395					2.7	204	82
2,6-Dimethoxy-*p*-cymene	1396			0.3			194	179
Longifolene	1398	0.7	0.7	0.2			204	105
$C_{15}H_{24}$	1400			0.5			204	147
$C_{15}H_{24}$	1404	0.2	0.2	1.3	2.2	1.3	204	93
$C_{15}H_{24}$	1407					0.1	204	105
α-Santalene	1409	0.2	0.2				204	94
$C_{15}H_{24}$	1418	**5.5**	**4.4**	0.2	0.1		204	161
γ-Patchoulene	1423	**4.5**	**4.9**	0.2			204	122
$C_{15}H_{24}$	1424					0.3	204	81
$C_{15}H_{24}$	1425				0.1		204	93
$C_{15}H_{24}$	1432	0.4	0.5				204	69
$C_{15}H_{22}$	1433			0.1			202	91
$C_{15}H_{22}$	1439	0.2	0.1	0.7			202	91

Table 3b. (continued)

α-Humulene	1440				0.6	0.3	204	93
$C_{15}H_{24}$	1442	3.0	3.0				204	41
$C_{15}H_{24}$	1443	0.2	0.1			0.1	204	105
$C_{15}H_{24}$	1443	0.5	0.5				204	107
$C_{15}H_{24}$	1444				0.3		204	69
$C_{15}H_{24}$	1445			2.9		**10.9**	204	105
$C_{15}H_{24}$	1450				0.2		204	93
$C_{15}H_{22}$	1451	0.2	0.1				202	159
β-Ionone	1454				0.1		192	177
$C_{15}H_{24}$	1454				0.1		204	43
$C_{15}H_{24}$	1457	0.1	0.1	0.3		0.1	204	81
$C_{15}H_{24}$	1462	1.2	0.9				204	105
ar-Curcumene	1464			0.7	1.6	0.8	202	119
α-Guaiene	1469	0.1	0.1	0.1			204	93
$C_{15}H_{24}$	1471	0.4					204	69
$C_{15}H_{24}$	1472			0.3			204	161
$C_{15}H_{24}$	1473		0.4		0.4		204	119
Pacifigorgiol isomer	1473					1.3	222	110
$C_{15}H_{24}$	1476			0.3	1.1		204	161
$C_{15}H_{24}$	1479	0.1	0.2	0.4		1.8	204	93
$C_{15}H_{24}$	1479	0.3	0.3				204	161
γ-Elemene	1482	0.4	0.4				204	81
$C_{15}H_{24}$	1486				0.2	0.2	204	93
α-Bulnesene	1489	0.7	0.5	0.2			204	107
$C_{15}H_{24}$	1492		0.2				204	119
Bornyl isovalerate	1492	0.1	0.1	0.1	0.1	0.5	238	95
$C_{15}H_{26}O$	1494	0.2	0.2	0.4	0.4	0.5	222	69
$C_{15}H_{24}$	1495		0.1	0.2	0.3	0.2	204	93
β-Gurjunene	1498	0.9	0.9	0.6	0.7	0.4	204	122
Kessane	1507		0.3	0.4		0.6	222	43
δ-Cadinene	1507	0.4			0.5		204	161

Table 3b. (continued)

Component	Retention index[a]	Sample 34[b]	35	36	37	38	M[c]	Bp[d]
$C_{15}H_{26}O$	1522	0.6	0.6		2.4		222	59
Pacifigorgiol	1523			2.6		2.1	222	110
Faurinone	1526					0.2	222	43
$C_{15}H_{24}$	1527	0.2	0.2	0.3	0.5	0.5	204	93
Myrtenyl isovalerate	1535	0.2			3.0	0.9	236	91
$C_{15}H_{24}$	1535	0.2	0.3	0.6		0.9	204	121
$C_{15}H_{26}O$	1536					1.8	222	123
Maaliol	1541	0.9	1.1				222	43
$C_{15}H_{26}O$	1548	2.1	2.1	2.7	0.2	**5.3**	222	91
$C_{15}H_{24}O$	1552	0.7	0.7	1.2	2.0		220	41
$C_{15}H_{26}O$	1555					2.0	222	121
$C_{15}H_{22}O$	1556	1.6	1.6		0.4		218	91
Citronellyl isovalerate	1558			2.7		0.6	240	81
$C_{15}H_{24}O$	1564	2.0	1.5		0.2		220	41
$C_{15}H_{26}O$	1565	2.4	2.4	0.8	0.3	0.6	222	69
$C_{15}H_{26}O$	1568					0.9	222	59
Ledol	1575	2.4		1.6	0.7	1.0	222	43
$C_{15}H_{24}O$	1578		2.3				220	93
$C_{15}H_{24}O$	1591					0.5	220	135
$C_{15}H_{26}O$	1592	**5.8**	2.0	0.8	0.4		222	43
$C_{15}H_{24}O$	1597			2.8	**5.0**		220	119
Bornyl hexanoate	1600			0.5			252	95
$C_{15}H_{26}O$	1602			2.5	2.2		222	43
$C_{15}H_{22}$	1603					0.1	202	159
$C_{15}H_{26}O$	1604	0.3				1.3	222	121
$C_{15}H_{26}O$	1604	0.4	0.4				222	43
$C_{15}H_{24}O$	1605					0.8	220	69
γ-Eudesmol	1605					1.5	222	161
β-Eudesmol	1613		0.5	**7.9**			222	59

Table 3b. (continued)

$C_{15}H_{26}O$	1617	1.0	0.6		2.7		222	43
Valerianol	1625			**19.9**	**11.5**	2.1	222	161
Patchouli alcohol	1625	**6.0**	**8.6**				222	83
$C_{15}H_{26}O$	1635				0.8		222	59
Valeranone	1639	1.0	0.8	2.5	0.5	**16.4**	222	98
$C_{15}H_{26}O$	1645			1.0	0.8	0.7	222	82
***epi*-α-Bisabolol**	1659			1.7	0.8	**7.4**	222	69
$C_{15}H_{24}O$	1674	2.3	1.9				220	43
$C_{17}H_{28}O_2$ (isovalerate)	1677			0.9	0.4	0.2	264	57
Valerenal	1688			3.5	0.4	**4.7**	218	91
$C_{15}H_{24}O$	1692	0.8	0.8	0.2	0.1	0.2	220	43
Valerenol (*cis* or *trans*)	1698			1.6	0.2	1.4	220	91
Valerenal isomer	1701	**14.8**	**18.5**				218	91
Eugenyl isovalerate	1728			0.3	0.4		248	164
$C_{17}H_{28}O_2$ (acetate)	1746	0.2	0.2	3.2			264	75
Drimenol	1750	0.5	0.6				222	109
$C_{17}H_{28}O_2$ (acetate)	1768		0.5	0.2	0.5		264	43
α-Kessyl acetate	1772				0.2		280	43
cis-Valerenyl acetate	1777	1.3	0.9	0.6		0.6	262	43
trans-Valerenyl acetate	1796	0.2				0.5	262	43
Isoeugenyl isovalerate	1814			0.2		0.3	248	164
Kessanyl acetate	1827	0.1	0.1				280	43
Valerenic acid	1831	1.0	1.2		0.5	0.6	234	91
$C_{17}H_{26}O_2$ (acetate)	1867	0.6	0.4	0.9	0.1		262	43
$C_{17}H_{32}O_3$ (isovalerate)	1881			1.2			284	57
Cryptofauronyl acetate	1890	0.1	0.1	0.9	0.2	0.6	280	43
Palmetic acid	1946	0.1	0.1	0.2	0.1	0.2	256	60
cis-Valerenyl isovalerate	1984			0.1	0.1	0.2	304	57
Patchoulyl acetate	2001	**3.3**	**3.1**				264	43
trans-Valerenyl isovalerate	2024	0.1	0.1	1.9	0.1	1.8	304	57

[a]Retention Index relative to C_9-C_{21} *n*-alkanes on the CP-Sil 5 column; [b]tr = trace (<0.05%);[c] M = molecular weight; [d]BP = base peak (100%).

Chapter 5

COMPOSITION OF THE ESSENTIAL OIL FROM ROOTS AND RHIZOMES OF *VALERIANA WALLICHII* DC.[5]

Introduction

Valeriana wallichii DC. (synonym *V. jatamansi* Jones), family Valerianaceae, grows abundantly in the temperate zones of the Himalayas, from Kashmir to Bhutan at an altitude of 1300-3300 m, in the Khasia Hills (India) at an altitude of 1300-2000 m, and in Afghanistan and Pakistan (Sood, 1965; Rashid *et al.*, 1972; Blatter *et al.*,1988; Hölzl, 1996). Local names of the plant are 'tagar' (Hindi) and 'tagara' (Sanskrit); its English name is Indian or Pakistani valerian.

V. wallichii is a perennial, erect herb; 60-100 cm high. The roots are yellowish-brown, 1.5-7 cm long and 1-2 mm thick. The rhizome is yellowish to brownish, 4-7 cm long and 1 cm thick, subcylindrical. Leaves are radical, persistent, stalked, cordate-ovate, acute, toothed. The flowers are white or tinged with pink in a terminal corymb 2.5-8 cm across, often unisexual, and the male and female on different plants. Fruits are small, smooth, without hairs. The market samples of the rhizomes are unbranched. The odour is valerianous, and the taste bitter and camphoraceous (Kapoor, 1990).

V. wallichii has long been in use in Ayurvedic and Unani systems of medicine (Gupta and Shah, 1981). The whole plant and the roots are applied in Ayurveda for nervous debility and failing reflexes, as a hypnotic, and to treat spastic disorders like chorea and gastrospasms. Roots are described to be useful in hysteria, epilepsy, shell shock, and neurosis (Kapoor, 1990).

The essential oil yield has been reported to vary from 0.3% to 2.0% (Sood, 1965; Anonymous, 1992b). The oil and the resinoid are used for flavour and fragrance purposes, but the oil has also some antibacterial and antifungal

[5]This Chapter is based on:
Bos R, Woerdenbag HJ, Hendriks H, Malingré ThM (1992) Der indische oder pakistanische Baldrian, *Valeriana wallichii* DC. (=*Valeriana jatamansi* Jones). *Z. Phytother.* **13**, 26-34.
Bos R, Woerdenbag HJ, Hendriks H, Smit HF, Wikström HV, Scheffer JJC (1997) Composition of the essential oil from roots and rhizomes of *Valeriana wallichii* DC. *Flavour Fragr. J.* **12**, 123-131.

activities (Girgune *et al.*, 1980). Secondary metabolites found so far in *V. wallichii* are two alkaloids (chatinine and valerine), aliphatic acids, several steroids, flavonoids (e.g. linarin), phenols, tannins, saponins, sugars, valepotriates, and a naphtholic acid (Bos *et al.*, 1992; Pande and Shukla, 1993).

In Western phytotherapy, the roots and rhizomes of *V. wallichii* are used for the preparation of phytomedicines with a mild sedative action, like those of *V. officinalis* L. *s.l.* and *V. edulis* Nutt. ex Torr. & Gray ssp. *procera* (H.B.K.) F.G. Meyer (Bos *et al.*, 1994; 1997a).

In view of an increasing demand for *V. wallichii*, its supply cannot be met only by collection from wild sources. Therefore, cultivation on a commercial scale has started in Uttar Pradesh, India, and also in Germany (Gupta and Shah, 1981; Wienschierz, 1978)

In this chapter, we describe the analysis of the essential oils isolated from roots and rhizomes of *V. wallichii* grown in Germany and The Netherlands, and from commercially available plant material of various origin.

Experimental

Plant material

Roots and rhizomes of *V. wallichii* were obtained from a local market in Kathmandu (Nepal) in 1993, from the botanical garden of the University of Groningen, from two experimental gardens in The Netherlands (at Buitenpost and Elburg), from the botanical garden of the University of Münster and from that at Weihenstephan (Germany), from a herbal company in The Netherlands (Verenigde Nederlandse Kruidencoöperatie ua, Elburg), and from five importers in Germany (H. Carroux, Hamburg; H. Ambrosius, Hamburg; M.C.M. Klosterfrau, Köln; P. Müggenburg, Hamburg; and Finzelberg's Nachfolger, Andernach). Voucher specimens have been deposited at the Department of Pharmaceutical Biology, University Centre for Pharmacy, Groningen, The Netherlands.

Isolation procedure

Each essential oil sample was isolated as described in chapter 4; 10.0 g of dry and ground root material were used as to the commercially obtained samples of plant material.

In addition, several oil samples were separated into two fractions - with hydrocarbons and oxygen-containing compounds, respectively - by eluting 250 µl of oil on a Bakerbond SPE column, filled with 1 g of silica gel (J.T. Baker, Deventer, The Netherlands), with subsequently 5 ml of *n*-hexane and 5 ml of diethyl ether. After gentle evaporation of the solvents of both fractions, 50 µl of each residue were diluted with 950 µl of cyclohexane and then submitted to GC and GC-MS analysis.

Gas chromatography and gas chromatography-mass spectrometry

GC and GC-MS analysis were performed as described in chapter 4.

Also a Unicam 610/Automass 150 GC-MS system was used. The GC conditions were: column, WCOT fused-silica CP-Sil 5 CB (25 m x 0.25 mm i.d., film thickness, 0.25 µm; Chrompack); oven temperature programme, 50-290°C at 4°C/min; injector temperature, 260°C; carrier gas, helium; inlet pressure, 5 psi; linear gas velocity, 32 cm/s; split ratio, 20:1; injected volume, 1.0 µl. MS conditions: ionization energy, 70 eV; ion source temperature, 250°C; interface temperature, 280°C; scan speed, 2 scans/s; mass range, 34-500 u.

The identity of the components was assigned by comparison of their retention indices, relative to C_9-C_{21} *n*-alkanes, and mass spectra with corresponding data from reference compounds and from the literature (Adams, 1989; Tucker and Maciarello, 1993). The percentages of the components were calculated from the GC peak areas, using the normalization method.

Results and discussion

The essential oil contents of the investigated samples varied between 0.09% and 1.30% (v/w), calculated on a dry weight basis (Table 1). In the literature, essential oil contents between 0.3% and 2.0% for the roots and rhizomes of *V. wallichii* are mentioned (Sood, 1965; Anonymous, 1992b).The low oil contents of the Nepalese material (sample 8), as well as of the other commercially available samples (9-16) may be due to their age and probably also to improper drying and storage. Nothing is known about the origin, the collection method, and the time between collecting, drying and analysing of these samples. The essential oil contents of the freshly collected samples from The Netherlands and from Germany (samples 1-7) varied between 0.13% and 1.30%. Only these

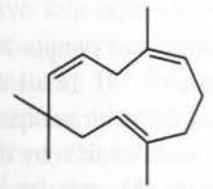
Table 1. Essential oil content of freshly obtained plant material (1-6), and of commercial samples (7-16) of roots and rhizomes of *Valeriana wallichii* DC.; the content (%) was calculated on a dry weight basis

Sample	Origin	%
1	Botanical garden, Groningen (roots)	0.55
2	Botanical garden, Groningen (rhizomes)	1.30
3	Experimental garden, Buitenpost	0.78
4	Experimental garden, Elburg	0.17
5	Botanical garden, Münster	0.90
6	Botanical garden, Weihenstephan	0.72
7	VNK	0.13
8	Market, Kathmandu	0.20
9	Carroux, Hamburg	0.26
10	H. Ambrosius, Hamburg	0.47
11	M.C.M. Klosterfrau, Köln	0.30
12	M.C.M. Klosterfrau, Köln	0.19
13	P. Müggenburg, Hamburg	0.23
14	P. Müggenburg, Hamburg	0.37
15	P. Müggenburg, Hamburg	0.17
16	Finzelberg 's Nachfolger, Andernach	0.09

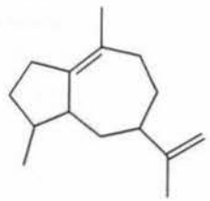
samples were submitted to distillation immediately after collection and subsequent drying for 48 h at 36 °C. The ratio between the oil content of the roots and that of the rhizomes of the samples from Groningen (samples 1 and 2) was ca 1:2.5. This is in agreement with results reported for the subterranean parts of *V. officinalis*, where almost the same ratio was found (Eisenhuth, 1956).

There were only small differences between the composition of the oils of the commercially available samples (8-16). The main components were γ-patchoulene, α-humulene (**39**), α-bulnesene (**40**), bornyl isovalerate, patchouli alcohol, and two unidentified components with a retention index (RI) of 1431 and 1645, respectively. In the Nepalese oil, also an unknown sesquiterpene (RI = 1443) was one of the main components. In the European samples 1-6, α-santalene (**41**), γ-patchoulene, the unknown sesquiterpene $C_{15}H_{24}$ (RI = 1431), *ar*-curcumene (**42**) and xanthorrhizol (**43**) were the main components. Patchouli alcohol was the dominant component of sample 7.

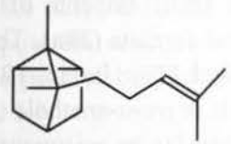
Furthermore, there was a large difference in the oil composition between the roots and the rhizomes from the plants grown in Groningen (samples 1 and 2). In the root oil the main monoterpenes were *p*-cymene and terpinolene, and the main sesquiterpenes α-santalene, the unknown $C_{15}H_{24}$ (RI



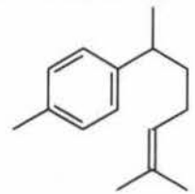
39 α-Humulene



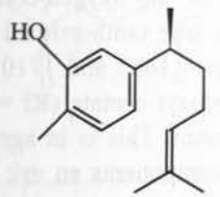
40 α-Bulnesene



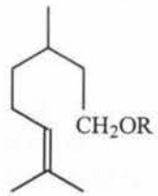
41 α-Santalene



42 *ar*-Curcumene



43 Xanthorrhizol



44 Citronellyl isovalerate
R = $COCH_2CH(CH_3)_2$

= 1431), *ar*-curcumene and xanthorrhizol. In the rhizome oil camphene, γ-patchoulene, α-humulene, bornyl isovalerate, maaliol and xanthorrhizol were the major components.

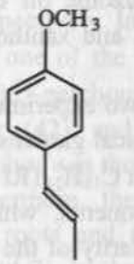
In the oils from the plants obtained from the two experimental gardens in the Netherlands, and in the oils from the two botanical gardens in Germany (samples 3-6), α-santalene, γ-patchoulene, the unkown $C_{15}H_{24}$ (RI = 1431), *ar*-curcumene and xanthorrhizol were the main components, while patchouli alcohol was present in small amounts only. The similarity of the composition of the oils 1-6 suggests that these samples belong to one strain of *V. wallichii*,

perhaps once imported to Europe by a botanical garden, from where it was distributed to other locations.

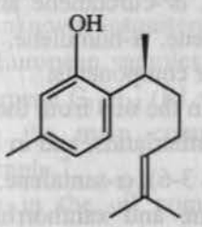
There was a rather great similarity between the oil of sample 7 and the oils from the commercially available samples (samples 8-16). In all these oil samples, except in sample 15, patchouli alcohol was the main component. In some oil samples (9, 12-15) large amounts of bornyl isovalerate were detected, together with smaller amounts of citronellyl isovalerate (**44**; samples 8, 9, 12, 15) and α-kessyl isovalerate (**24b**; samples 12, 13, 15). In the literature only one study was found in which bornyl isovalerate was the main component (30.0%) of the oil of *V. wallichii*, together with small amounts of bornyl acetate (1.1%), and traces of borneol (**30**) and bornyl formate (**30a**). The total sesquiterpene hydrocarbons content in that study was 27% (Jurcic, 1974). In the samples 12, 14 and 15 we detected small amounts of *trans*-anethole (**45**). In contrast to *cis*-anethole, *trans*-anethole is not considered to be poisonous.

The compound with the RI of 1645 (samples 1-6, 9-16) was present at a maximum concentration of 13%. With NICI-MS (*m/z* = 221, $[M-H]^-$) we confirmed its formula to be $C_{15}H_{26}O$, i.e. that of an oxygen-containing sesquiterpene. Also three sesquiterpene alcohols, viz. one xanthorrhizol isomer (RI = 1723) and two curcuphenol (**46**) isomers (RIs = 1685 and 1710), were detected in most of the samples. Probably xanthorrhizyl acetate (RI = 1767) and curcuphenyl acetate (RI = 1738) were also present. This is in agreement with the NICI-mass spectra, that showed for both components an *m/z* of 259 ($[M-H]^-$), and an *m/z* fragment of 59 (CH_3COO^-).

Another unknown compound with an RI of 1555 was found; its formula was confirmed the be $C_{15}H_{26}O$ using NICI-MS (*m/z* = 221, $[M-H]^-$). After isolation of the same compound from a commercial sample of *Nardostachys jatamansi* oil, using low-pressure column chromatography and



45 *trans*-Anethole



46 Curcuphenol

preparative thin-layer chromatography, we could tentatively identify it as a tertiary sesquiterpene alcohol with a valerane skeleton. Spectral data: $[\alpha]^{20}_D$: + 23.8° (MeOH); UV (MeOH) λ_{max} 216 nm; IR ν, cm^{-1}: 3610 and 3490 (OH); 3080, 1630 and 880-900 (C=C); 1100 (tert. OH); ^{1}H-NMR ($CDCl_3$, TMS): δ 4.82, 4.67 (d, 2H, H-15), 1.47 (s, 1H, OH), 1.20 (s, 3H, H-14), 0.89, 0.87, 0.85, 0.83 (q, 6H, H-12, H-13); ^{13}C-NMR ($CDCl_3$, TMS): δ 153.5 (C, C-6), 108.4 (CH_2, C-15), 82.3 (C, C-5), 52.9 (C, C-10), 40.3 (CH, C-3), 33.1 (CH, C-11), 27.6 (CH_3, C-14), 20.1 (CH_3, C-13), 19.2 (CH_3, C-12), 40.6, 39.2, 38.2, 35.3, 31.2, 18.4 (CH_2, C-1, C-2, C-4, C-7, C-8, C-9). Based on these spectral data, we propose the structure as presented in Figure 1. The mass spectra of some sesquiterpene alcohols discussed above, or the acetates thereof, are given in Figures 2-6.

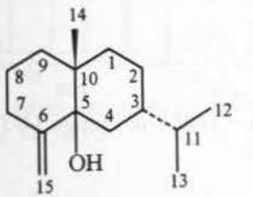


Figure 1. Proposed structure of the tertiary sesquiterpene alcohol with an RI of 1555

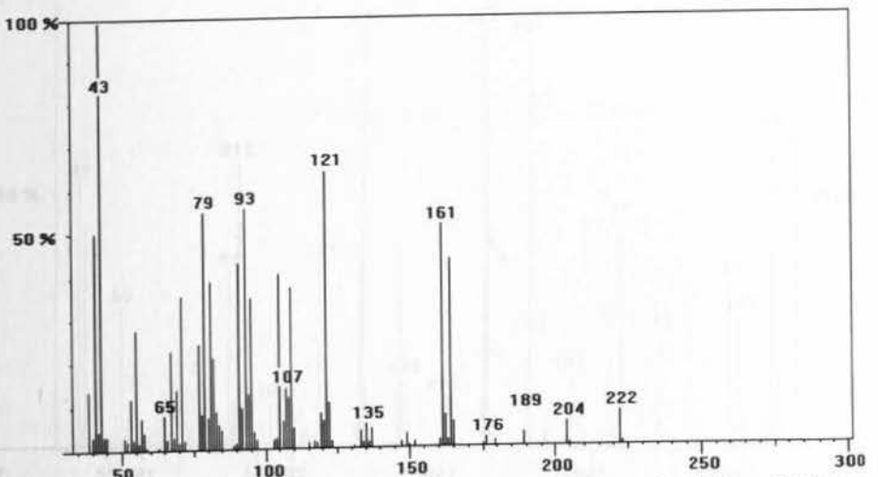


Figure 2. Mass spectrum of the sesquiterpene alcohol with a retention index of 1555; *m/z* range 34-300 u

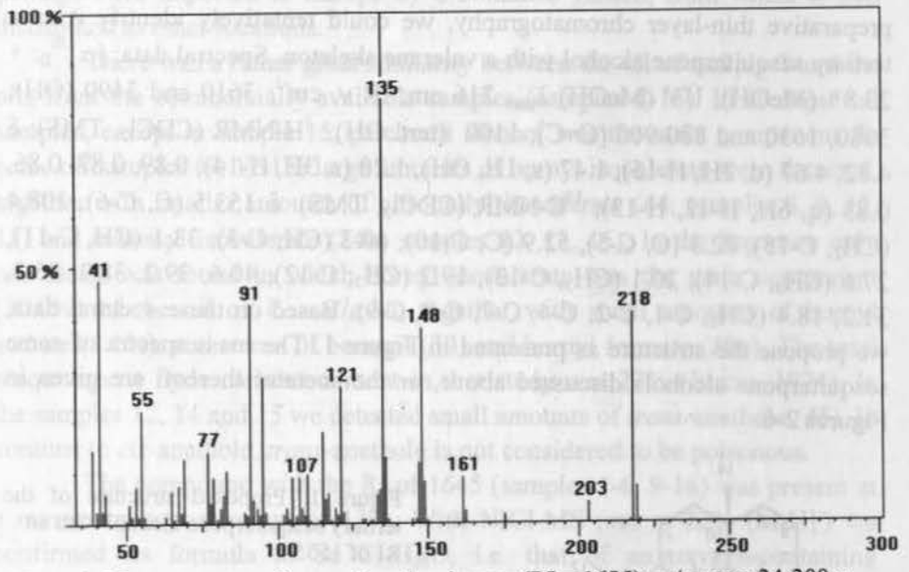


Figure 3. Mass spectrum of curcuphenol or isomer (RI = 1685); *m/z* range 34-300 u

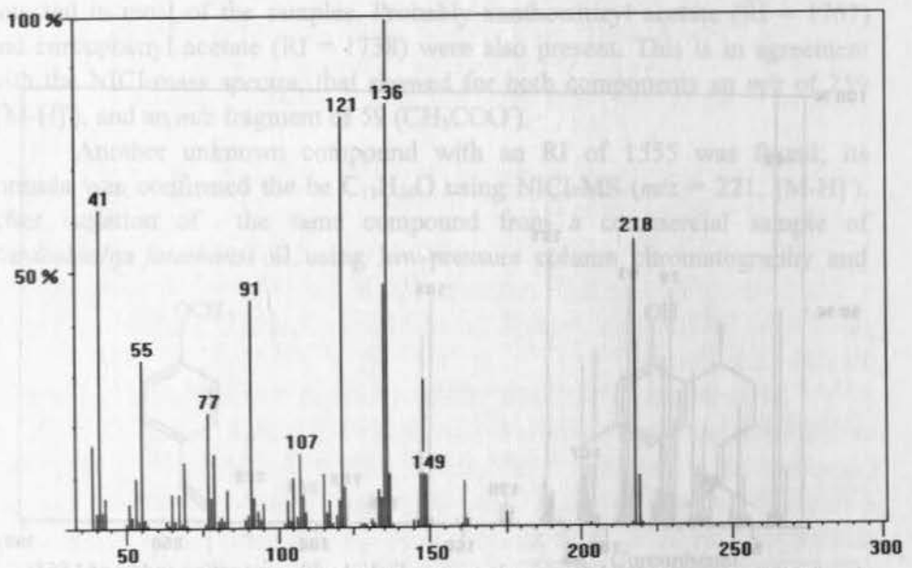


Figure 4. Mass spectrum of a xanthorrhizol isomer (RI = 1723); *m/z* range 34-300 u

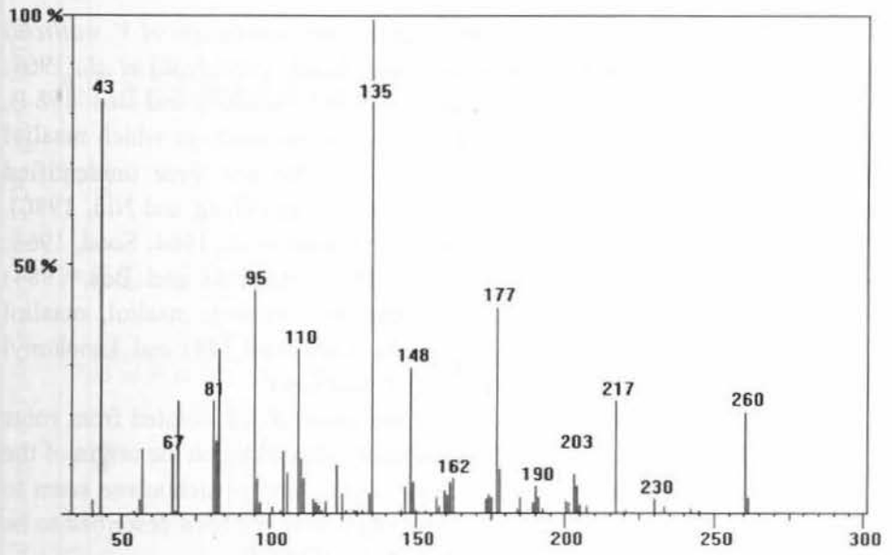


Figure 5. Mass spectrum of the sesquiterpene acetate with a retention index of 1738; *m/z* range 34-300 u

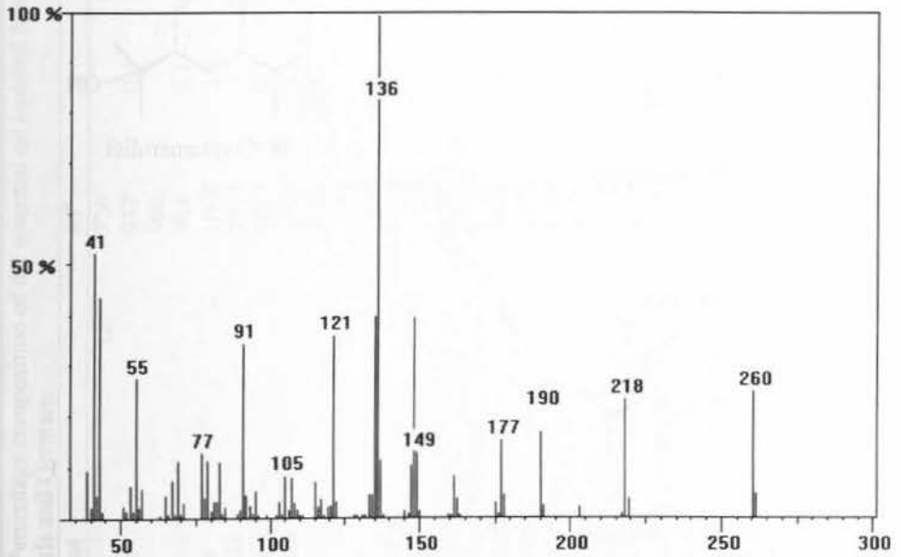
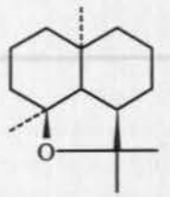


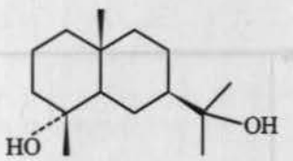
Figure 6. Mass spectrum of the sesquiterpene acetate with a retention index of 1767; *m/z* range 34-300 u

In the literature available with regard to the essential oil of *V. wallichii* (Kulkarni *et al.*, 1964; Narayanan *et al.*, 1964; Sood, 1964; Joshi *et al.*, 1968; Paknikar and Kirtany, 1972; Wang and Niu, 1980; Hendriks and Bos, 1984), only data on its oil content are given, except for one study in which maaliol was found (11.6%) together with α-pinene (14.5%) and three unidentified components (10.9%, 18.7% and 20.3%, respectively) (Wang and Niu, 1980). Other investigators (Kulkarni *et al.*, 1964; Narayanan *et al.*, 1964; Sood, 1964; Joshi *et al.*, 1968; Paknikar and Kirtany, 1972; Hendriks and Bos, 1984) mentioned the well-known components patchouli alcohol, maaliol, maaliol oxide (**47**), valeranone, cryptomeridiol (**48**), kanokonol (**49**) and kanokonyl acetate together with some sesquiterpene hydrocarbons.

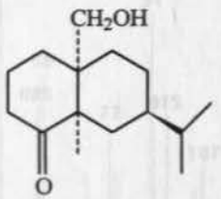
In conclusion, the composition of the essential oil isolated from roots and rhizomes of *V. wallichii* varied considerably depending on the origin of the plant material. However, patchouli alcohol, and β- and γ-patchoulene seem to be characteristic for this oil, as these compounds have not been described to be present in other *Valeriana* species, such as *in V. officinalis*.



47 Maaliol oxide



48 Cryptomeridiol



49 Kanokonol

Table 2. Percentage composition of the essential oil isolated from roots and rhizomes of *Valeriana wallichii* DC. grown in The Netherlands and Germany

Component	RI[a]	1	2	3	4	5	6	7	M[b]	BP[c]
α-Pinene	926		0.2	0.2	0.3	0.2	0.1	0.3	136	93
Camphene	937	0.4	2.5			0.2	0.1		136	93
Sabinene	960					tr[d]			136	93
β-Pinene	964	0.1	0.1			0.1	0.1		136	93
p-Cymene	1008	2.3	tr			tr			134	119
1,8-Cineole	1015		tr						154	43
Limonene	1017	0.3	0.1			0.2	0.1		136	68
Terpinolene	1075	2.8							136	93
Linalol	1082	0.7	0.3			tr			154	71
Camphor	1112		tr						152	95
Borneol	1140		0.5	0.2	tr	0.1			154	95
Terpinen-4-ol	1154		0.1	0.1	0.1	0.1	0.1		154	71
α-Terpineol	1165		0.1	0.1	0.1	0.1			154	59
Thymol methyl ether	1207		0.2						164	149
Carvacrol methyl ether	1211		0.1						164	149
Bornyl acetate	1264	0.1	0.6	0.4	0.1	0.4	0.2	0.6	196	95
Thymyl acetate	1276		0.1						192	135
Carvotanacetone	1299			0.1	0.1	0.1			152	110
δ-Elemene	1326		0.2	0.9	0.5	0.5	1.2		204	121
Geranyl acetate	1360		0.2		0.1				196	43
β-Patchoulene	1370		1.4						204	161
2,5-Dimethoxy-*p*-cymene	1372	0.4	0.1	0.3	0.1	0.3	0.3		194	179
β-Elemene	1379		0.3			0.1		0.2	204	68
2,6-Dimethoxy-*p*-cymene	1396	0.3	0.2	0.5	0.3	0.4	0.3		194	179
α-Cedrene	1398	0.4	0.2	0.2	0.1	0.2	0.3		204	93
α-Santalene	1409	**5.6**	**0.9**	**4.5**	**2.4**	**3.1**	**4.5**		204	94
γ-Patchoulene	1423	**3.7**	**4.4**	**3.4**	**1.6**	**3.0**	**3.3**	**2.6**	204	122
$C_{15}H_{24}$	1431	**26.8**	**2.3**	**25.1**	**15.1**	**19.2**	**25.3**	**0.2**	204	93

Table 2. (continued)

Component	RI[a]	1	2	3	4	5	6	7	M[b]	BP[c]
α-Humulene	1440		3.6			0.1	0.2	0.5	204	93
$C_{15}H_{24}$	1443	2.3	1.1	1.1	0.4	1.0	2.0		204	107
2-Phenylethyl isovalerate	1457		tr	0.2	0.3	0.2			206	104
***ar*-Curcumene**	1464	**7.0**	**1.1**	**10.3**	**4.3**	**12.7**	**11.6**		202	119
α-Guaiene	1469	0.3	0.3	0.5	0.4	0.5	0.4		204	93
$C_{15}H_{24}$	1473	0.6	0.6	0.8	0.8	0.7	0.7		204	161
$C_{15}H_{24}$	1478		0.7						204	189
γ-Elemene	1482		1.3				0.3		204	81
α-Bulnesene	1489	0.2	3.0	0.4	0.2	0.5	0.4		204	107
Bornyl isovalerate	1492	0.6	2.6	0.8	0.5	1.0	0.9	0.2	238	95
β-Gurjunene	1498	1.0	1.2	0.9	1.0	0.9	1.0		204	122
Kessane	1507	0.4	1.0		0.2	0.2	0.4	2.2	222	43
δ-Cadinene	1507			0.4	0.3	0.4			204	161
cis-Nerolidol	1514	0.8	0.5	0.7			1.2		222	69
Selinene isomer	1515		0.1						204	93
$C_{15}H_{24}O_2$ (isovalerate)	1522							1.2	236	91
$C_{15}H_{24}$	1527	0.8	0.2	0.4	0.1	0.6	0.5		204	91
$C_{15}H_{26}$	1538							4.3	206	110
Maaliol	1541		2.3		0.2	0.2	0.1		222	43
$C_{15}H_{26}O$	1548		0.5	0.2	0.4	0.4	0.1		222	107
Thymyl isovalerate	1552		0.3					1.0	234	135
$C_{15}H_{26}O$	1555	0.8	1.1	0.9	1.5	1.0	0.9		222	43
Turmerol	1561			0.1	1.6	0.2			218	132
2-Phenylethyl hexanoate	1564	0.6	1.6	1.0	1.6	1.1	0.9	1.4	222	104
$C_{16}H_{24}O_2$ (hexanoate)	1571	0.2	0.3		0.3		0.1		248	119
Ledol	1575	0.2	0.7	0.6	0.8	0.6	0.4		222	41
$C_{15}H_{26}O_2$ (isovalerate)	1585		0.6	0.2	1.4	0.4		0.4	238	69
Bornyl hexanoate	1600	1.1	0.4	1.3	1.8	1.6	1.4		252	95
$C_{15}H_{26}O$	1616	0.2	0.2	0.3	0.2	0.2	0.2		222	119
Patchouli alcohol	1625	**0.4**	**0.4**	**0.3**	**0.5**	**0.7**	**0.5**	**48.8**	222	83

Table 2. (continued)

Valeranone	1639	1.1	1.2	1.4	1.7	1.4	1.2	1.2	222	98
$C_{15}H_{26}O$	1645	1.1	1.2	1.6	1.4	2.0	1.7		222	82
$C_{15}H_{20}O_2$	1655	1.8	1.9	1.7	1.8	2.0	1.9		232	150
$C_{15}H_{22}O$ (curcuphenol or isomer)	1685	0.7	0.8	1.8	1.1	2.0	1.3		218	135
$C_{15}H_{22}O$ (valerenal isomer)	1701	1.0							218	91
$C_{15}H_{22}O$ (curcuphenol or isomer)	1710	0.9	1.4	1.3	0.8	1.7	1.9	0.4	218	135
Xanthorrhizol	1717	**8.2**	**9.8**	**16.7**	**12.2**	**18.4**	**12.6**		218	136
Xanthorrhizol isomer	1723							0.9	218	136
$C_{17}H_{24}O_2$ (curcuphenyl acetate?)	1738		0.5	0.3	0.1		0.5		260	135
Drimenol	1750							0.1	220	109
$C_{17}H_{24}O_2$ (xanthorrhizyl acetate?)	1767	0.7	1.4	0.2	0.1		1.5		260	136
α-Kessyl acetate	1772					0.2			280	43
$C_{15}H_{26}O$	1823			0.1	0.5	0.2			222	123
$C_{15}H_{24}O_2$	1826	2.1	2.1	0.1	0.5		0.6		236	82
$C_{15}H_{24}O_2$	1856	0.5	0.5	0.6	0.7	0.7	0.7		236	123
Cryptofauronyl acetate	1890	0.2	tr		0.1				280	43
$C_{16}H_{22}O_2$	1915							0.2	246	147
$C_{15}H_{24}O_2$	1939	1.9	1.3	0.3	1.4	0.4	1.2		236	123
Patchoulyl acetate	2001	0.2			1.0			1.5	264	43
Total		81.8	62.9	83.5	63.1	82.5	85.2	68.2		
Grouped components										
Monoterpene hydrocarbons		5.9	2.9	0.2	0.3	0.7	0.4	0.3		
Oxygen-containing monoterpenes		3.4	6.9	4.9	5.4	5.0	4.5	3.4		
Sesquiterpene hydrocarbons		48.7	22.7	48.0	26.7	43.0	50.5	7.8		
Oxygen-containing sesquiterpenes		21.4	26.9	27.5	27.0	30.5	27.0	55.3		
Others		2.4	3.5	2.9	3.7	3.3	2.8	1.4		

[a]Retention Index relative to C_9-C_{21} *n*-alkanes on the CP-Sil 5 (25 m x 0.32 mm) column; [b]M = molecular weight; [c]BP = base peak (100%); [d]tr = trace (<0.05%).

Table 3. Percentage composition of the essential oil isolated from commercially available samples of dried roots and rhizomes of *Valeriana wallichii* DC.

Component	RI[a]	8	9	10	11	12	13	14	15	16
α-Pinene	926	0.2	0.1	0.1	0.2	0.1	0.1	0.2	0.8	1
Camphene	937	0.3					0.1	2.4	0.9	0.1
Sabinene	960	0.2								
β-Pinene	964	0.2						0.1	0.2	
β-Myrcene	980	0.1								
p-Cymene	1008	tr[b]							tr	
1,8-Cineole	1015	0.2							tr	
Limonene	1017	0.1	0.1				0.1	0.1	tr	
Linalol	1082	0.1	0.1			0.4	0.1	0.3	1	
Camphor	1112						0.1		tr	
Borneol	1140	0.1	0.5	0.1	0.4	1.3	0.8	0.5	1.7	0.6
Terpinen-4-ol	1154			tr		0.4	0.1	0.1	0.4	0.2
α-Terpineol	1165	0.1	0.1			0.3	0.2	0.1	0.3	
1,4-Dimethoxybenzene	1171								0.1	0.1
Thymol methyl ether	1207	0.2	0.1	0.1		0.4	0.1	0.2	0.5	0.1
Carvacrol methyl ether	1211	0.1				0.2	0.1	0.1	0.3	tr
Thymol methyl ether isomer	1222								0.1	
trans-Anethole	1249					0.1		0.1	0.4	
Bornyl acetate	1264	0.1	0.6	0.1	0.6	1.7	1.5	0.6	1.9	1.7
Thymyl acetate	1276						0.1	0.1	0.1	
δ-Elemene	1326	0.1	0.3	0.1		0.7	1.1	0.2	0.8	0.8
Citronellyl acetate	1332		0.2			0.6	0.1		0.1	
Geranyl acetate	1360		0.6		0.1	1.2	0.4	0.2		
Benzyl isovalerate	1366			0.1					0.6	
β-Patchoulene	1370	0.6	0.5	0.8	1.3	0.2	0.3	1.5		0.5
2,5-Dimethoxy-*p*-cymene	1372	0.3	0.3	0.4	0.1		0.2			
β-Elemene	1379	0.5			0.1			0.3	0.1	0.4

Table 3. (continued)

2,6-Dimethoxy-p-cymene	1396	0.4	0.3	0.1	0.1	0.3	0.2	0.2	0.5	
α-Santalene	1409	0.9	0.8	0.9	1.0	1.1	1.6	0.9	2.9	0.8
$C_{15}H_{24}$	1417	0.1	0.3	1.0	1.7		0.3	0.2	0.4	
γ-Patchoulene	1423	**10.2**	**3.4**	**5.1**	**4.5**	**2.2**	**3.1**	**4.4**		**4.2**
$C_{15}H_{24}$	1431	**0.8**	**2.3**	**2.3**	**2.2**	**2.6**	**6.0**	**2.3**	**7.0**	**3.5**
α-Humulene	1440	**5.6**	**1.1**	**3.9**	**2.3**	**0.7**	**1.2**	**3.7**		**2.1**
$C_{15}H_{24}$	1443	14.0								
2-Phenylethyl isovalerate	1457	0.1	0.9			2.1	1.3	0.4	2.3	0.9
ar-Curcumene	1464	0.4	2.0	0.9	0.8	1.2	2.7	1.1	1.7	1.5
α-Guaiene	1469	0.4		0.3	0.2		0.4	0.3	0.1	
$C_{15}H_{24}$	1473	1.0	0.4	0.7	0.4		0.3	0.6		
$C_{15}H_{24}$	1478	1.1	0.5	0.7	0.4	0.2	0.4	0.7		0.3
γ-Elemene	1482	1.6	0.9	1.3	0.8	0.4	0.6	1.3		1.4
α-Bulnesene	1489	**5.3**	**5.0**	**3.1**	**1.0**	**1.1**	**1.3**	**3.1**	**1.0**	**1.5**
Bornyl isovalerate	1492	**0.2**	**6.0**	**0.2**	**0.5**	**9.5**	**4.5**	**2.6**	**11.9**	
α-Terpenyl isovalerate	1498	3.0								
β-Gurjunene	1498	0.8	0.8	1.4	0.8	0.3	0.7	1.2		1.3
Kessane	1507	0.4	1.2	1.9	0.6	0.8	1.2	1.0	1.3	1.5
Selinene isomer	1515		0.1				tr	0.1	0.3	0.1
$C_{15}H_{24}O_2$ (isovalerate)	1522		0.2	0.2	0.4	0.2	0.3	0.2	0.2	
$C_{15}H_{24}$	1527	0.1								
$C_{15}H_{26}O$	1527		0.3	0.1	0.2		0.1	0.2	0.4	0.3
$C_{15}H_{26}$	1538		0.8		8.2			2.2		
Maaliol	1541	2.9								
trans-Nerolidol	1542		4.0	5.1		10.5	6.0		1.0	3.4
$C_{15}H_{26}O$	1548	2.7								
Thymyl isovalerate	1552	0.8	0.5		0.4	0.9	0.5	0.3	1.1	
$C_{15}H_{26}O$	1555			0.4				1.1		
Citronellyl isovalerate	1558	2.0	2.0			2.1	0.5		4.4	
Turmerol	1561	0.8								
2-Phenylethyl hexanoate	1564	3.1	0.5	1.6	2.2	1.3	3.1	1.5	0.5	

Table 3. (continued)

Component	RI[a]	8	9	10	11	12	13	14	15	16
$C_{16}H_{24}O_2$ (hexanoate)	1571	0.2								
Ledol	1575	1.7								
$C_{15}H_{26}O_2$ (isovalerate)	1585	0.4	0.9	0.4	0.5	0.9	0.3	0.6	1.5	
Bornyl hexanoate	1600	0.4	0.9	0.8	1.1	2.3	1.8	0.4	0.7	
$C_{15}H_{26}O$	1616	0.9								
Patchouli alcohol	1625	**15.0**	**31.9**	**49.4**	**47.0**	**12.2**	**14.4**	**43.0**	**1.4**	**25.3**
Valeranone	1639	2.6	1.0	0.8	1.8	1.3	0.6	0.7	0.3	0.5
$C_{15}H_{26}O$	1645		**7.2**	**0.2**	**4.2**	**5.6**	**4.3**	**2.2**	**13.3**	**3.0**
$C_{15}H_{20}O_2$	1655		0.4			0.3	0.7		0.4	
$C_{15}H_{22}O$ (curcuphenol or isomer)	1685	0.3								
$C_{15}H_{22}O$ (valerenal isomer)	1697	0.2								
$C_{15}H_{22}O$ (curcuphenol or isomer)	1710	0.1	0.5			0.5	0.6			
Xanthorrhizol	1717	0.2	1.7	0.2	0.3	0.3	4.5	0.1	0.4	
Xanthorrhizol isomer	1723		0.4	0.2	0.4	0.5	0.3	0.3	0.4	
Drimenol	1750						0.1		0.2	
$C_{17}H_{24}O_2$ (xanthorrhizyl acetate?)	1767	0.1	0.1							
α-Kessyl acetate	1772	0.6								
$C_{15}H_{24}O$	1826	0.5								
$C_{16}H_{22}O_2$	1915		3.1			7.8	3.0	1.5	7.8	
$C_{15}H_{24}O_2$	1939	0.4								
α-Kessyl isovalerate	1986			0.3		1.6	2.0	0.5	4.6	
Patchoulyl acetate	2001	2.0				0.4	1.6		0.2	
α-Kessyl hexanoate	2070					1.0	0.4	0.2	0.8	
Total		87.8	85.9	85.3	86.8	79.8	76.4	86.2	79.3	57.1
Grouped components										
Monoterpene hydrocarbons		1.1	0.2	0.1	0.2	0.1	0.3	2.8	1.9	1.1
Oxygen-containing monoterpenes		8.7	13.6	2.5	4.2	23.5	13.0	6.8	26.7	3.4
Sesquiterpene hydrocarbons		43.4	18.9	22.4	25.7	10.0	18.9	23.9	14.3	17.6
Oxygen-containing sesquiterpenes		31.4	51.8	58.6	54.5	42.8	39.8	50.8	32.5	34.0
Others		3.2	1.4	1.7	2.2	3.4	4.4	1.9	3.9	1.0

[a]Retention Index relative to C_9-C_{21} *n*-alkanes on the CP-Sil 5 column; [b]tr = trace (<0.05%).

Chapter 6

THE ESSENTIAL OIL AND VALEPOTRIATES FROM ROOTS OF *VALERIANA JAVANICA* BLUME GROWN IN INDONESIA[6]

Introduction

In Indonesia, the roots of *Valeriana javanica* Blume (synonym *V. hardwickii* Wall. (Hegnauer, 1973; Engel, 1976); local name 'akar valerian'), Valerianaceae, are used as a mild tranquilizer (Sugati and Hutapea, 1991). This is the same application as that of *V. officinalis* L. *s.l.*, *V. wallichii* DC. and *V. edulis* Nutt. ex Torr. & Gray ssp. *procera* (H.B.K.) F.G. Meyer in Western phytotherapy (Bos *et al.*, 1994; 1997a). In addition, the roots of *V. javanica* are traditionally used in Indonesia as an insect repellant and as a 'devil repellant' after birth (Sugati and Hutapea, 1991). *V. javanica* is also found in the temperate zones of the Himalayas, from Kashmir to Bhutan at an altitude of 1300-3300 m, and in the Khasia Hills (India) at an altitude of 1300-2000 m (Blatter *et al.*, 1988). In India this *Valeriana* species is regarded as a good substitute for *V. wallichii* and *V. officinalis* (Blatter *et al.*, 1988). So far, phytochemical data on *V. javanica* are not available.

Experimental

Plant material

V. javanica originated from Tawangmangu, Solo (Central Java), Indonesia, 1200 m above sea level. The roots were collected in June 1994, during the dry season. A voucher specimen has been deposited at the Medicinal Plant Research Station (Kebun Hortikultura) in Tawangmangu.

Isolation procedure

The essential oil was isolated as described in chapter 4. Part of the oil sample was fractionated as described in chapter 5.

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[6]This chapter is based on:
Bos R, Woerdenbag HJ, Hendriks H, Sidik, Wikström HV, Scheffer JJC (1996) The essential oil and valepotriates from roots of *Valeriana javanica* Blume grown in Indonesia. *Flavour Fragr. J.* **11**, 321-326.

Chromatographic procedures

GC analysis of the essential oil was performed as described in chapter 4. GC-MS (EI) was performed on a Unicam 610/Automass 150 GC-MS system. The GC-MS conditions were as described in chapter 5.

The identity of the components was assigned by comparison of their retention indices, relative to C_9-C_{19} *n*-alkanes, and mass spectra with corresponding data from reference compounds and from the literature (Adams, 1989; Tucker and Maciarello, 1993). The percentages of the components were calculated from the GC peak areas, using the normalization method.

Valerenic acid and derivatives, and valepotriates were isolated, and subsequently analysed by HPLC, as described in chapter 3.

Results and discussion

The air-dried roots of *V. javanica* yielded 0.36% (v/w) essential oil. In Table 1 the chemical composition of the essential oil is shown. More than 50 components, mainly oxygen-containing sesquiterpenes (59%), amounting to more than 85% of the total oil, were identified unambiguously. The main constituent of the oil was α-kessyl acetate; other major constituents included α-cedrene, patchouli alcohol and kessane, while valeranone and bornyl acetate were also found to be present in a relative amount of more than 1%.

In addition to the list of compounds presented in Table 1, several unidentified sesquiterpene alcohols with M = 220 or M = 222, as well as unknown constituents with M = 218 or M = 216 were found in trace amounts (< 0.05%).

Despite the presence of some kessane compounds, we were unable to detect kessoglycol or its mono- and di-acetates in the fraction with oxygen-containing compounds, using single ion monitoring.

The presence of patchoulenes (retention index [RI] 1370, 1408 and 1423) may be explained by a partial decomposition of patchouli alcohol (RI = 1625) during the process of hydrodistillation (Bos *et al.*, 1992).

The peak with an RI of 1277 showed a distinct mass spectrum (Fig. 1a), but we were unable to identify this compound by comparing it with spectral data bases. In addition, the mass spectrum of the peak with an RI of 1414 is given (Fig. 1b). This compound is an as yet unidentified sesquiterpene; a compound with the same fragmentation pattern but with an RI of 1400 was found in the oil of *V. officinalis* (Bos *et al.*, 1997c; chapter 4).

Table 1. Percentage composition of the essential oil isolated from roots of *Valeriana javanica* Blume

Component	RI[a]	%[b]	M[c]	BP[d]
α-Pinene	926	0.3	136	93
Camphene	937	0.5	136	93
Sabinene	960	0.2	136	93
β-Pinene	964	0.2	136	93
p-Cymene	1008	0.2	134	119
1,8-Cineole	1015	0.7	154	43
Limonene	1017	0.7	136	68
cis-Linalol oxide (furanoid structure)	1053	0.1	170	59
trans-Linalol oxide (furanoid structure)	1067	0.1	170	59
Terpinolene	1075	tr	136	93
Linalol	1082	0.7	154	71
Camphor	1112	tr	152	41
cis-1,2-Limonene oxide	1122	tr	152	41
trans-1,2-Limonene oxide	1125	tr	152	41
Nerol oxide	1134	tr	170	68
Borneol	1140	0.7	154	95
Terpinen-4-ol	1154	0.2	154	71
p-Cymen-8-ol	1161	0.1	150	43
Isodihydrocarvone	1165	0.1	152	67
p-Menth-1-en-9-al	1199	tr	152	79
Cumin aldehyde	1203	0.1	148	133
Thymol methyl ether	1207	0.5	164	149
Carvacrol methyl ether	1211	0.2	164	149
Thymol methyl ether isomer	1216	tr	164	149
Carvacrol methyl ether isomer	1222	tr	164	149
Geraniol	1233	0.3	154	69
Bornyl acetate	1264	**2.7**	196	43
Unknown	1277	0.5	162	77
Thymol	1290	0.1	150	135
Carvacrol	1299	0.1	150	135
δ-Elemene	1326	0.1	204	43
β-Patchoulene	1370	0.3	204	161
β-Bourbonene	1372	tr	204	81
2,5-Dimethoxy-*p*-cymene	1372	0.3	194	179
β-Elemene	1379	0.1	204	68
$C_{15}H_{22}$	1392	0.1	202	159
α-Cedrene	1398	**14.8**	204	41
$C_{15}H_{24}$	1404	0.2	204	189
α-Patchoulene	1408	0.1	204	107
$C_{15}H_{24}$	1414	tr	204	147
Dimethoxy-*p*-cymene isomer	1420	tr	194	179

Table 1. (continued)

Component	RI[a]	%[b]	M[c]	BP[d]
γ-Patchoulene	1423	1.6	204	122
Dimethoxy-*p*-cymene isomer	1425	tr	194	179
Calacorene	1433	0.1	200	157
$C_{15}H_{24}$	1437	0.7	204	41
α-Humulene	1440	0.8	204	41
ar-Curcumene	1464	0.1	202	119
Calamenene	1496	0.1	202	159
Kessane	1507	**6.8**	222	43
δ-Cadinene	1507	0.3	204	161
Faurinone	1526	tr	222	43
$C_{15}H_{26}O$	1536	0.1	222	43
$C_{15}H_{26}O$	1549	0.4	222	159
β-Eudesmol	1613	0.5	220	59
Patchouli alcohol	1625	**10.3**	222	83
Guaiol	1627	tr	222	59
Valeranone	1639	**3.2**	222	41
α-Kessyl alcohol	1660	0.8	238	43
Eugenyl isovalerate	1728	tr	248	164
Drimenol	1750	0.3	222	109
α-Kessyl acetate	1772	**36.2**	280	43
$C_{17}H_{28}O_2$ (acetate)	1778	0.1	262	43
Tridensenone	1783	0.1	216	173
Total		87.8		
Grouped components				
Monoterpene hydrocarbons		2.1		
Oxygen-containing monoterpenes		7.0		
Sesquiterpene hydrocarbons		19.4		
Oxygen-containing sesquiterpenes		58.8		
Others		0.5		

[a]Retention Index relative to C_9-C_{19} *n*-alkanes on the CP-Sil 5 column; [b]tr = trace (<0.05%); [c]M = molecular weight; [d]BP = base peak (100%).

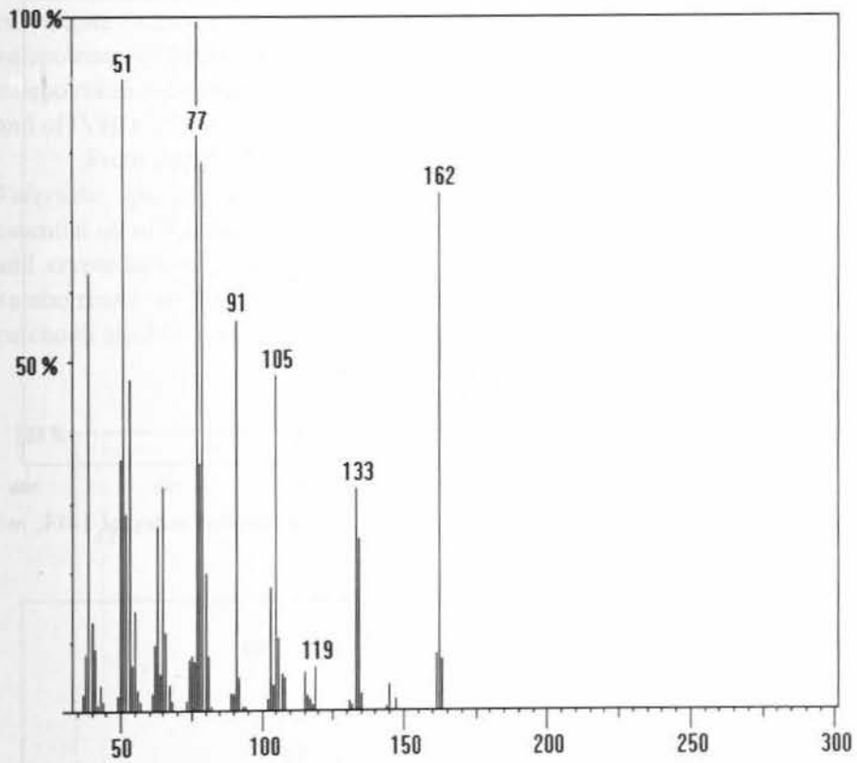

Figure 1a. Mass spectrum of unknown component with a retention index of 1277; *m/z* range 34-300 u

Figure 2 shows the mass spectra of kessane (RI = 1507), α-kessyl alcohol (RI = 1660), and α-kessyl acetate (RI = 1772). A great similarity is seen in the mass fragmentation patterns of these components. Based on the similarity of the mass spectra of some trace components (RI = 1796, 1812 and 1826; not shown in Table 1) having M = 236 or M = 238 with those of the identified kessanes, we suppose that they are probably esters of kessane isomers. Some other trace components might be derivatives of 14-hydroxyvaleranone (**49**, = kanokonol).

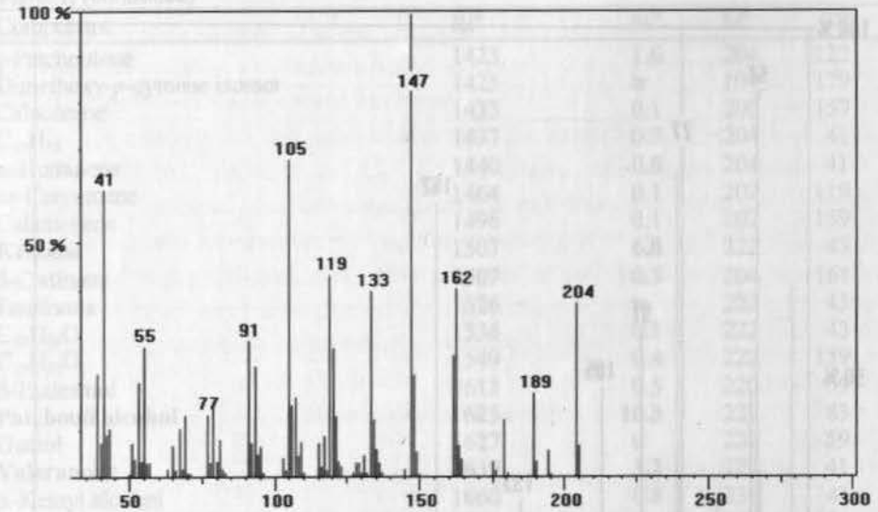

Figure 1b. Mass spectrum of unknown component with a retention index of 1414; *m/z* range 34-300 u

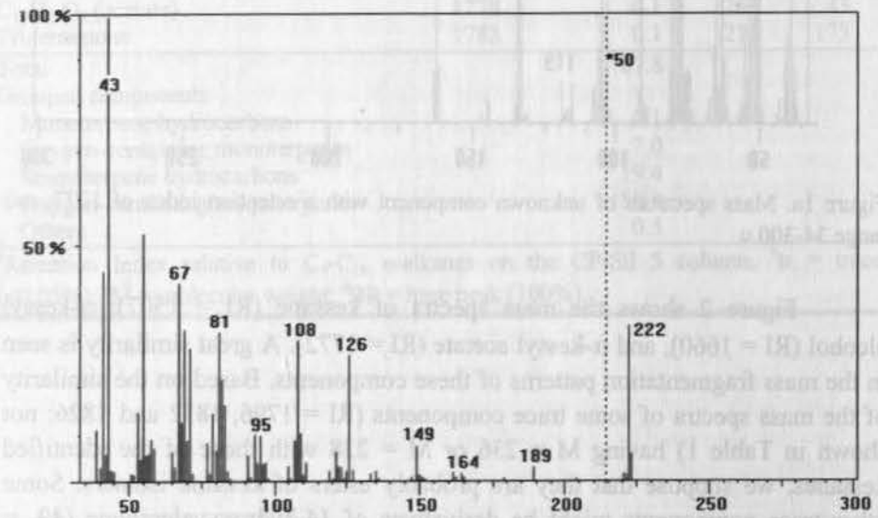

Figure 2a. Mass spectrum of kessane (RI = 1507); *m/z* range 34-300 u; the spectrum was extended 50 times in the *m/z* range 215-300u

The total valepotriate content was 2.96% based on dry weight, consisting of valtrate (2.24%), isovaltrate (0.60%), acevaltrate (0.11%) and an unknown valepotriate (0.01%), all of which were detected at 255 nm. No monoene valepotriates were detected at 200 nm (the absorption maximum of didrovaltrate and of IVHD. Valerenic acid and its derivatives were not detected either.

From a phytochemical point of view, *V. javanica* does not resemble the *Valeriana* species used in Western countries. The main constituents of the essential oil of *V. officinalis* are bornyl acetate, valeranone, valerenal, valerianol and cryptofauronol, and that of the oil of *V. wallichii,* grown in Europe, is xanthorrhizol. In the oil isolated from commercial root samples of *V. wallichii* patchouli alcohol was found to be a major component. *V. edulis* contains only

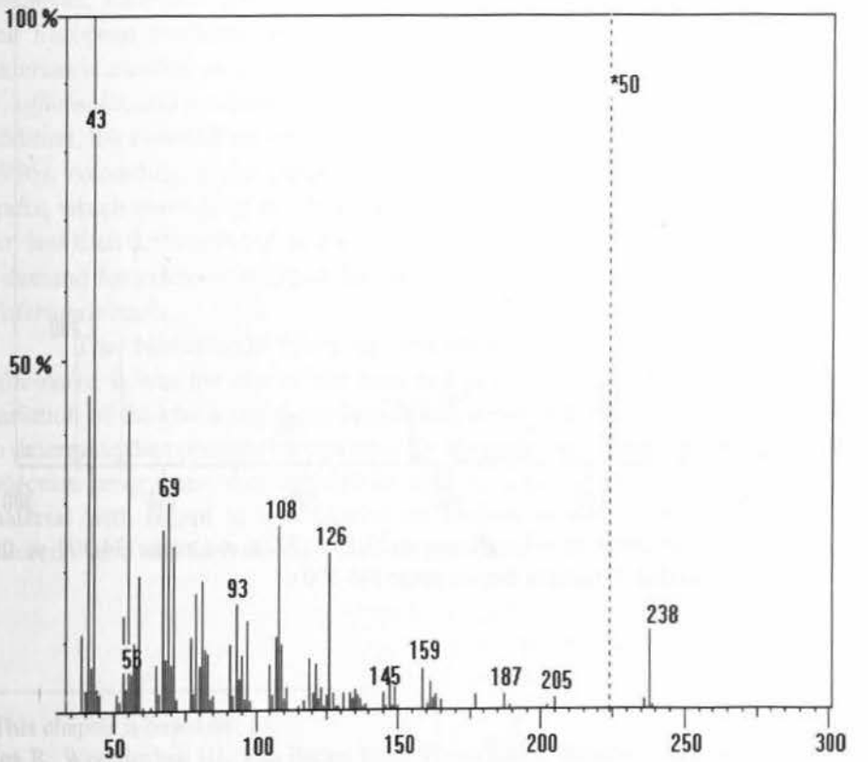

Figure 2b. Mass spectrum of α-kessyl alcohol (RI = 1660); *m/z* range 34-300 u; the spectrum was extended 50 times in the *m/z* range 224-300 u

very small amounts of essential oil (Bos *et al.*, 1997f).

Because of the presence of several kessane-like compounds in *V. javanica*, some phytochemical similarity exists with the Japanese species *V. fauriei* Briq. (Nishiya *et al.*, 1995; Oshima *et al.*, 1995).

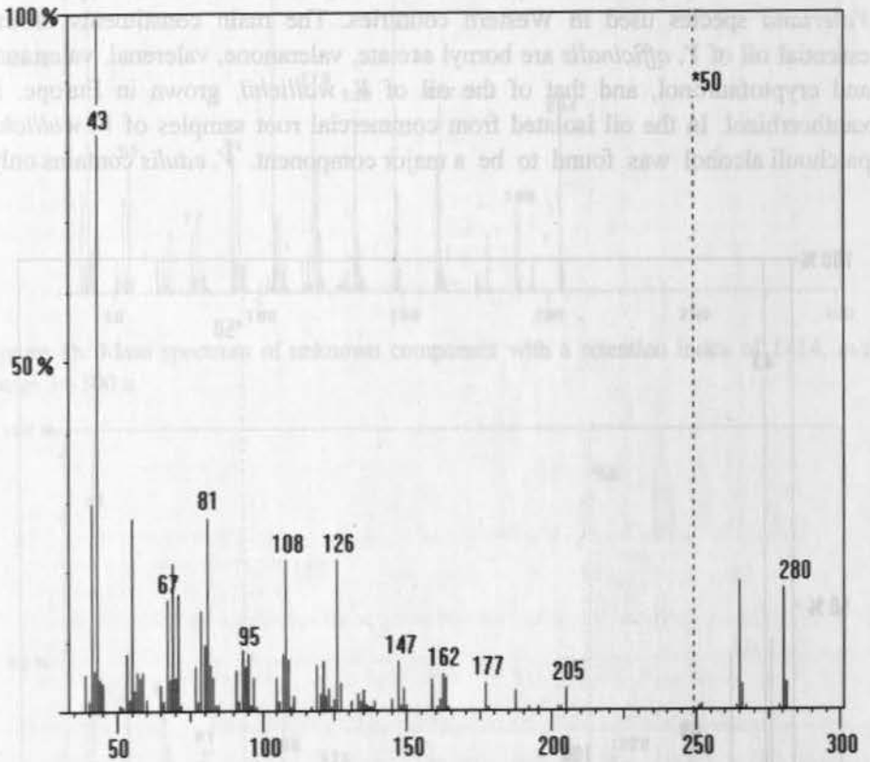

Figure 2c. Mass spectrum of α-kessyl acetate (RI = 1772); *m/z* range 34-300 u; the spectrum was extended 50 times in the *m/z* range 248-300 u

Chapter 7

SEASONAL VARIATIONS OF THE ESSENTIAL OIL, VALERENIC ACID AND DERIVATIVES, AND VALEPOTRIATES IN *VALERIANA OFFICINALIS* ROOTS AND RHIZOMES, AND THE SELECTION OF PLANTS SUITABLE FOR PHYTOMEDICINES[7]

Introduction

For the production of valerian based phytomedicines that are used as mild sedatives, *Valeriana officinalis* L. *s.l.*, Valerianaceae, is the species included in the European Pharmacopoeia (Anonymous, 1997). The biological activity of valerian is ascribed to valerenic acid and derivatives, which are characteristic of *V. officinalis*, and to valepotriates, that are also found in other valerian species. In addition, the essential oil may contribute to the sedative effect (Bos *et al.*, 1994; 1996). According to the European Pharmacopoeia, the crude drug *Valerianae radix*, which consists of the dried roots and rhizomes of *V. officinalis*, contains not less than 0.5% (v/w) of essential oil. It is expected that in the near future also a demand for valerenic acid and derivatives will be included in the monograph on *Valerianae radix*.

The Netherlands plays an important role in the cultivation of *V. officinalis*. It was the aim of the present study to get insight into the seasonal variation of the characteristic groups of secondary metabolites of this plant, and to determine the optimum harvest time for the roots and rhizomes. Furthermore, a selection programme was initiated in order to improve the quality of the plant material with regard to its essential oil content as well as to its contents of valerenic acid and derivatives, and of valepotriates.

[7]This chapter is based on:
Bos R, Woerdenbag HJ, Van Putten FMS, Hendriks H, Scheffer JJC (1997) Seasonal variation of the essential oil, valerenic acid and derivatives, and valepotriates in *Valeriana officinalis* roots and rhizomes, and the selection of plants suitable for phytomedicines. *Planta Med.*, in press.

Materials and methods

Plant material

Seeds of *V. officinalis* L. *s.l.* were obtained from the Verenigde Nederlandse Kruidencoöperatie ua (VNK), Elburg, The Netherlands. In all experiments, seeds were sown in a glass house in the second half of March, and the small plants were transferred in the open in the second half of May, in an experimental garden of VNK in Elburg. The soil was sandy, with a pH of 5.6. The planting distance was 50 x 30 cm. Voucher specimens of the plants from the various experiments have been deposited at the Department of Pharmaceutical Biology, University Centre for Pharmacy, Groningen.

Seasonal variation

In the season 1989-1990, *V. officinalis* plants were investigated for their contents of essential oil, valerenic acid, acetoxyvalerenic acid, valtrate and isovaltrate. One field was sown in the spring of 1989, and the plants were harvested from August 1989 until April 1990. Another field was sown in the spring of 1988, and the plant material was harvested from April 1989 until February 1990. Once a fortnight, ten plants were collected for analysis.

In the spring of 1990, 1991 and 1992, plants were raised as described above. Harvesting of the subterranean parts was started in August of the year in which the seeds were sown, and continued until April of the subsequent year. In all cases, once a fortnight ten plants were collected; subsequently the subterranean parts were washed, dried for 10 days at room temperature, and then investigated for their contents and composition of essential oil, valerenic acid and derivatives, and valepotriates.

Selection of plant material

In the spring of 1989, seeds of five *V. officinalis* strains, 8545-1, 8570-1, 8570-3, 8545-pl and 8570-pl, selected from earlier breeding experiments (in the early 1980s) by VNK, were sown. Plants were harvested mid September of the same year, and the subterranean parts were investigated for their contents and composition of essential oil, valerenic acid and derivatives, and valepotriates.

In the spring of 1990, seeds of plants from the five strains, which were selected from the 1989 experimental field, were sown and the plants were earned in September of the same year. A total of 445 samples was analysed for their valerenic acid content. Of 64 samples selected on the basis of a high valerenic

acid content, the essential oil content was also determined and the composition of the oil investigated. For one strain, a valerenic acid content ≥0.4% was the criterion for selection, for the other four strains a content ≥0.3%.

In 1992 and 1993, respectively, 24 and 201 individually selected plants, based on the selection programmes of 1989 and 1990, were investigated. The essential oil content, and composition, the amount of valerenic acid and derivatives, and that of the valepotriates were determined. In addition, the extractable matter was determined.

Isolation of essential oil

Each essential oil sample was isolated as described in chapter 4.

Chromatographic procedures

GC and GC-MS analysis were carried out as described in chapter 4. Valerenic acid and derivatives, and valepotriates were analysed by HPLC as described in chapter 3.

Results and discussion

Seasonal variation

In order to get a first impression of the seasonal variations in essential oil, valerenic acid and derivatives, and valepotriates, subterranean parts of one-year and two-years old valerian plants were investigated in the season 1989-1990. One field was sown in spring 1989, the other field one year earlier, as described above. The essential oil content of the one-year old material ranged from 1.3% to 2.1% (v/w; based on dry weight) and was somewhat higher than that of the two-years old material, which ranged from 0.9% to 1.8%. The same pattern was observed for valerenic acid and derivatives, and for the valepotriates. The total content of valerenic acid and acetoxyvalerenic acid lay between 0.3% and 0.9% for the one-year old material and between 0.1% and 0.6% for the two-years old material. The total valtrate and isovaltrate content in the one-year old material varied between <0.1% and 1.1%, and in the two-years old material between 0.1% and 0.8%. In these experiments only a very low content (<0.05%), or even a complete absence, of hydroxyvalerenic acid was found, which is consistent with earlier findings (Hänsel and Schulz, 1982). The presence of hydroxyvalerenic acid is considered to be an artefact, resulting from improper drying procedures (Bos *et al.*, 1996).

In Figure 1, the variation in the essential oil content as well as in the

contents of valerenic acid and derivatives, and of valepotriates for the season 1989-1990 is shown for the plants sown in 1989. As can be seen, the highest essential oil content was found in September-November with a second maximum in March. The highest contents of valerenic acid and derivatives, and of the valepotriates were found in February and March, respectively; thereafter the contents of these compounds decreased again. These results are representative of the results obtained in all years under study, with only small variations from year to year. The composition of the essential oil remained more or less constant over the whole vegetation period (1989-1990), which was also representative of all years under study. The main constituents were valeranone (10-21%), crypto-fauronol (5-12%), valerenal (2-5%), valerianol (1-6%) and bornyl acetate (1-3%).

In order to get a more detailed insight into the seasonal variation of the characteristic secondary metabolites and with the aim of further selection, a more extended investigation programme was started, that lasted for three years. In early spring of each year 1990, 1991 and 1992, seeds of a *V. officinalis* strain were sown. Harvesting was started between mid August and the beginning of September of the first year, and was continued until the last week of April of the subsequent year. In Figure 2 the essential oil content for all years under study is

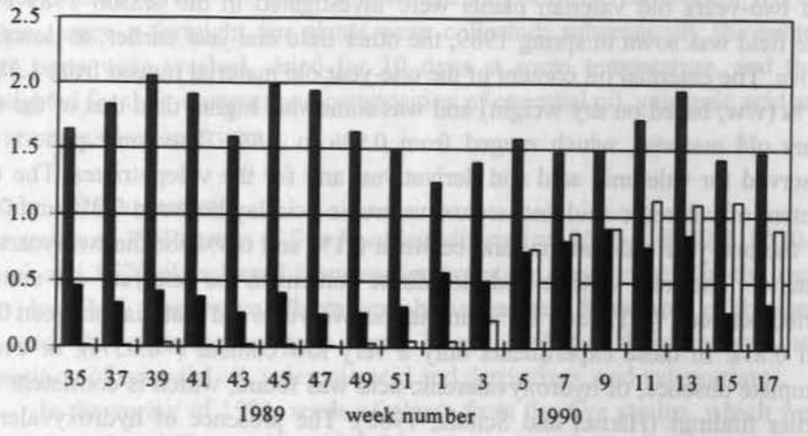

Figure 1. Seasonal variation of the contents of essential oil ▨, valerenic acid and derivatives ■, and valepotriates □ in *Valeriana officinalis*

shown. Despite marked variations from year to year, it can be concluded that maximum contents of essential oil in the subterranean parts of *V. officinalis* were found in September, ranging from 1.2% to 2.1%.

For valerenic acid and derivatives, and for the valepotriates the maximum contents were found in February-March: 0.7-0.9% for valerenic acid and derivatives (Figure 3) and 1.1-1.4% for the valepotriates (Figure 4).

Thus, when the content of valerenic acid and derivatives is considered the best-quality parameter for the drug, the plant should be harvested in February-March. For the essential oil content, the periods September-November and March are the best harvest times. Taking into account that harvesting of the subterranean parts in the winter period may give more difficulties, September may be preferred as the harvest time.

Selection of plant material

In continuation of a selection programme carried out in the early 1980s, that was mainly directed to the essential oil of valerian roots and rhizomes (Bos *et al.*, 1986a), we conducted a new and more extensive programme in order to improve the quality of the plant material not only with respect to the essential oil but also to valerenic acid and derivatives.

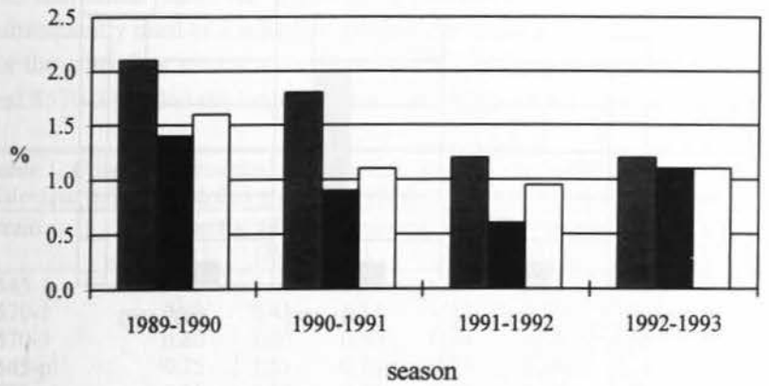

Figure 2. Content of essential oil in subterranean parts of *Valeriana officinalis* plants harvested in September ▨, January ■ and March □ of four subsequent seasons

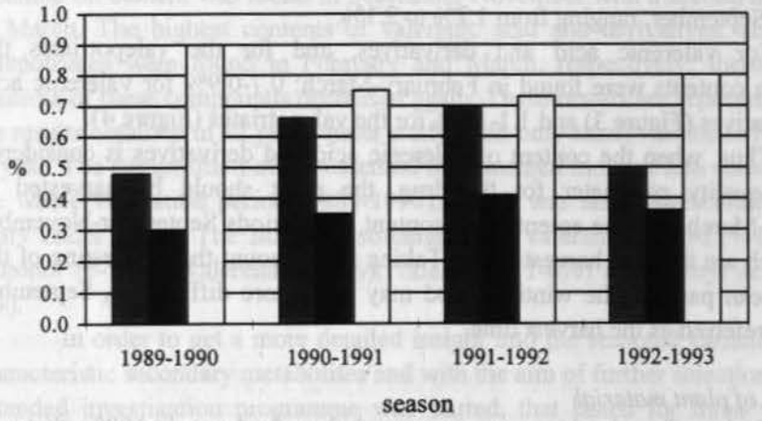

Figure 3. Content of valerenic acid and derivatives in subterranean parts of *Valeriana officinalis* plants harvested in September ■, January ■ and March □ of four subsequent seasons

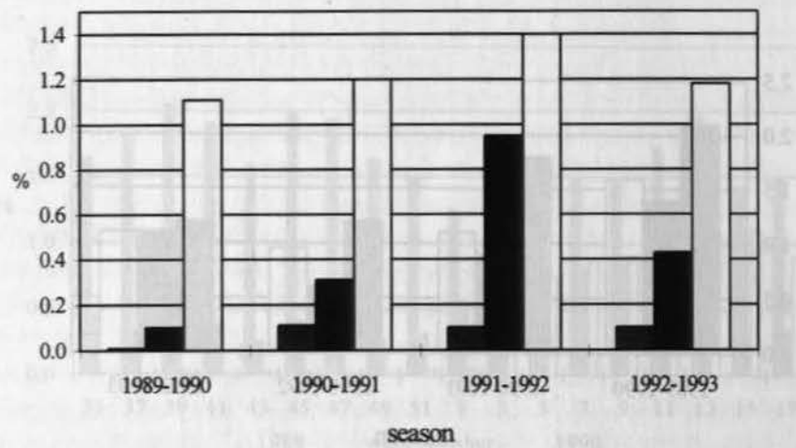

Figure 4. Content of valepotriates in subterranean parts of *Valeriana officinalis* plants harvested in September ■, January ■ and March □ of four subsequent seasons

The selection programme was started in 1989. After harvesting in mid September, the subterranean parts of five *V. officinalis* strains were investigated for their essential oil content, as well as for valerenic acid and derivatives, and valepotriates. In all cases a part of the roots and rhizomes was dried for 52 h at 50°C (VNK, Elburg), and another part was dried for 10 days at room temperature (about 20°C, University Centre for Pharmacy, Groningen). The results are given in Table 1.

Drying of the plant material at the higher temperature, which is the standard procedure at the experimental garden of VNK, resulted in a reduction of the essential oil content of up to 50%. In contrast, no marked differences were found in the content of valerenic acid and derivatives when both drying methods are compared. In the composition of the essential oils isolated from root material dried at room temperature and from the material dried at 50°C, large differences were not found, although there was a tendency of loss of a part of the most volatile monoterpenes after drying at the higher temperature. The valepotriate content of the material dried at room temperature was, with the exception of one strain, about 50% lower than that of the material dried at 50°C. During the 'slower' drying period the decomposition of valepotriates is likely to start. These compounds are known to be (thermo)labile, and they easily decompose, yielding baldrinals, in the presence of water (Bos *et al.*, 1996; chapter 3).

From plants of these five strains, seed was collected and sown in the spring of 1990. Harvesting took place in the autumn of the same year. A total of 445 individual plants was investigated for the content of valerenic acid that was subsequently used as a selection marker; for strain 8570-1 a content of 0.4%, and for the other four strains a content of 0.3% was the minimum. The strains 8545-1 and 8570-3 yielded the largest number of plants with a valerenic acid content ≥

Table 1. Contents of essential oil, valerenic acid and derivatives, and valepotriates of five *Valeriana officinalis* strains grown in 1989 (in %, based on dry weight)

Strain	Essential oil		Valerenic acids		Valerenic acid[a]		Valepotriates[b]	
	[1]	[2]	[1]	[2]	[1]	[2]	[1]	[2]
8545-1	1.21	1.69	0.31	0.36	0.51	0.55	0.05	0.03
8570-1	0.90	1.45	0.14	0.12	0.50	0.53	0.20	0.08
8570-3	0.80	1.67	0.40	0.44	0.58	0.65	0.15	0.10
8545-pl	0.75	1.51	0.16	0.15	0.24	0.22	0.02	0.02
8570-pl	1.01	2.13	0.37	0.23	0.53	0.57	0.10	0.04

[a]Total content of valerenic acid and acetoxyvalerenic acid; [b]total content of valtrate and isovaltrate;.[1]dried at 50°C for 52 h; [2]dried at room temperature (20°C) for 10 days.

0.3%: 19% and 17%, respectively. For the strains 8570-1 and 8545-pl this was 14% and 12%, respectively. Strain 8570-pl yielded the smallest number of plants with a high valerenic acid content: only 9%. In Table 2 the mean valerenic acid content of all 445 individual plants from the five strains, as well as the mean valerenic acid content and the mean essential oil content of the selected plants of the five investigated strains is given. No relationship was seen between the essential oil content and the content of valerenic acid, and the same was true for that of valerenal (data not shown). A high oil content was no guarantee for a high valerenic acid and/or valerenal content, and *vice versa*.

In 1992, 24 individual plants were examined for their essential oil, valerenic acid, and valepotriate contents. The mean essential oil content was 0.85% on a dry weight basis. The mean valerenic acid content was 0.45%, and the mean valepotriate content 0.74% (Table 3).

In September 1993, 201 individual plants selected from the five earlier mentioned strains were investigated for their contents of essential oil, valerenic acid as well as valepotriates. Also one of the criteria mentioned in the European Pharmacopoeia, namely the extractable matter (minimum 15%), was determined. The essential oil content lay between 0.38% and 1.28%, with a mean of 0.87%. Valeranone, one of the main components, was present in the oil in amounts between 4.3% and 20.2%. Valerianol (1.5%-10%) as well as cryptofauronol (0.5%-24.9%) and valerenal (3.7%-11%) were the other main components. In some of the oil samples investigated, there were only small quantitative differences with respect to valeranone, valerianol, cryptofauronol and valerenal. In these cases, it was not possible to indicate to what kind of 'chemotype' the plant belonged. We reported earlier that both the content of essential oil and its composition may strongly differ from plant to plant. In 1986, we distinguished four 'chemotypes', a valeranone, a valerenal, a cryptofauronol and an, at that time

Table 2. Mean value of the contents of valerenic acid and essential oil of five *Valeriana officinalis* strains grown in 1990 (in %, based on dry weight)

Strain	Valerenic acid[a]	Valerenic acid[b]	Essential oil[b]
8545-1	0.22 (n = 72)	0.43 (n = 14)	1.26 (n = 14)
8570-1	0.28 (n = 104)	0.45 (n = 15)	1.04 (n = 15)
8570-3	0.19 (n = 98)	0.41 (n = 17)	1.21 (n = 17)
8545-pl	0.19 (n = 91)	0.37 (n = 11)	1.03 (n = 11)
8570-pl	0.17 (n = 80)	0.38 (n = 7)	1.10 (n = 7)

[a]All plants; [b]plants with a valerenic acid content ≥ 0.4% (8570-1) or ≥ 0.3% (8545-1, 8570-3, 8545-pl and 8570-pl).

Table 3. Development of the contents of essential oil, valerenic acid, valepotriates and extractable matter of plants from five *Valeriana officinalis* strains during the breading experiments (in %, based on dry weight)

Year	Essential oil	Valerenic acid	Valepotriates	Extractable matter
1989[1]	1.69	0.26	0.05	n.a.
1990 (n=64)	1.13	0.41	n.a.	n.a.
1992 (n=24)	0.85	0.45	0.74	n.a.
1993 (n=201)	0.87	0.48	0.85	30.1

[1]Mean of the five strains; n.a. = not analysed.

unknown, sesquiterpene alcohol type. Meanwhile we have identified this sesquiterpene alcohol as being valerianol (Bos *et al.*, 1986a;1997c; chapter 4). Generally the bornyl acetate content was low, ranging from 0.8% to 6.5%. The valerenic acid content lay between 0.20% and 0.72%, with a mean of 0.48%. In only 3.5% of the investigated samples the valerenic acid content was less than 0.3%. The valepotriate content varied from 0.51% to 1.63%, with a mean of 0.85%. The extractable matter had a mean value of 30.1% and thus amply met the demand in the European Pharmacopoeia. The results of the experiments carried out between 1989 and 1993 are summarized in Table 3.

In conclusion, it can be stated that the selection procedures described in this chapter, yielded plant material harvested in September with a decreasing essential oil content, from 1.7% in 1989 to 0.87% in 1993, as well as an increasing content of valerenic acid, from 0.26% to 0.48% over the years. The relevance of breeding experiments becomes clear from the following data concerning the production of *Valerianae radix* in The Netherlands. In the season 1983-1984, 40 tons of *Valerianae radix* were harvested, in 1986-1987 30 tons, and over the period 1990-1994 only 50-60 tons. In the same time a large amount of plant material, mostly of low quality and very cheap, came from several countries in Eastern Europe. The last two years, however, *Valerianae radix* with a high quality is demanded, e.g. with a minimum essential oil content of 0.5% and a valerenic acid content of at least 0.3%. This quality can only be met by continuous and rigorous selection programmes. As a result, the production of *Valerianae radix* in The Netherlands rose to 120 tons in 1995; the expectation for 1996 was 130 tons, but due to the dry and windy spring, a part of the germinated seeds was blown away, so that only 80 tons were earned. The expectations for the next three years are as much as120, 150 and 200 tons, respectively.

Chapter 8

CYTOTOXIC POTENTIAL OF VALERIAN CONSTITUENTS AND VALERIAN TINCTURES[8]

Introduction

Valerian based phytomedicines are employed as antianxiety agents and sleep aid. For the preparation of these mild sedatives the subterranean parts of three *Valeriana* species, namely *V. officinalis* L. *s.l.*, *V. wallichii* DC. and *V. edulis* Nutt. ex Torr. & Gray ssp. *procera* (H.B.K.) F.G. Meyer, are commonly used. Considerable differences are found with respect to the pattern and content of secondary metabolites in the roots and rhizomes of these species. Consequently, also the various phytomedicines available on the market differ in composition (chapters 1 and 3).

Valepotriates and valerenic acid and derivatives thereof are considered as the active constituents of valerian, although this is not fully evidenced despite numerous pharmacological studies (for reviews see: Houghton, 1988; Morazzoni and Bombardelli, 1995; Bos *et al.*, 1997a). Valerenic acids, i.e. valerenic acid, acetoxyvalerenic acid, hydroxyvalerenic acid and methyl valerenate, are characteristic of *V. officinalis*, while valepotriates are also found in other *Valeriana* species (chapters 4 and 7).

Valepotriates possess alkylating properties, for which mainly the epoxy group is responsible. Several studies report on the cytotoxicity and mutagenicity of valepotriates in *in vitro* cell systems, and inhibition of DNA and protein synthesis in *in vitro* cultured mammalian cells have been described (Bounthanh *et al.*, 1981; 1983; Von der Hude *et al.*, 1985; 1986; Hänsel, 1990; 1992; Keochanthala-Bounthanh *et al.*, 1990; 1993). Cytotoxic and mutagenic effects have also been described for baldrinals (Bounthanh *et al.*, 1981; Braun *et al.*, 1986; Von der Hude *et al.*, 1986; Dieckmann, 1988).

The aim of the present study was to investigate the cytotoxic potential of characteristic valerian constituents in two *in vitro* cultured human tumour cell

[8]This chapter is based on:
Bos R, Hendriks H, Scheffer JJC, Woerdenbag HJ (1997/1998) Cytotoxic potential of valerian constituents and valerian tinctures. *Phytomedicine*, in press.

lines, and to compare the cytotoxicity of all compounds under similar experimental conditions. The compounds tested were valerenic acid, hydroxyvalerenic acid, acetoxyvalerenic acid and methyl valerenate, the valepotriates valtrate, isovaltrate, acevaltrate, didrovaltrate and IVHD, and their decomposition products baldrinal, homobaldrinal and isovaltral. Additionally, freshly prepared and stored tinctures, made from underground parts of *V. officinalis*, *V. edulis* and *V. wallichii*, were included in the tests. The cytotoxicity of these tinctures is evaluated in relation to their chemical composition.

Material and methods

Plant material

Subterranean parts of *V. officinalis* L. *s.l.* were obtained from the Verenigde Nederlandse Kruidencoöperatie ua, Elburg, The Netherlands. Subterranean parts of *V. wallichii* DC. and *V. edulis* Nutt. ex Torr & Gray ssp. *procera* (H.B.K.) F.G. Meyer were obtained from H. Finzelberg's Nachfolger GmbH & Co. KG, Andernach, Germany. Voucher specimens have been deposited at the Department of Pharmaceutical Biology, University Centre for Pharmacy, Groningen.

Test compounds

Valtrate, isovaltrate, acevaltrate and didrovaltrate were isolated from *V. edulis* ssp. *procera* and purified as described in chapter 3. Isovaleroxyhydroxy-didrovaltrate (IVHD) was obtained from LAT GmbH Dr. Tittel (Munich, Germany).

Baldrinal and homobaldrinal were prepared from valtrate and isovaltrate, respectively. Briefly, of each valepotriate 1 g was dissolved in 25 ml of ethanol-water 60:40 and left at room temperature for 2 weeks. After removal of the solvent under vacuum, the residue was taken up in hexane, while a dark-brown amorphous mass was left behind. The latter probably consisted of polymerization products. The hexane solution of the residue was subjected to preparative TLC using silica gel, with methylene chloride-ethyl acetate-acetone 48:1:1 as the mobile phase. This yielded baldrinal and homobaldrinal. Isovaltral was isolated as a decomposition product of isovaltrate (Denee *et al.*, 1979).

Valerenic acid and acetoxyvalerenic acid were isolated from *V. officinalis* as described in chapter 2. Hydroxyvalerenic acid was prepared by saponification of acetoxyvalerenic acid with a 10% solution of KOH in ethanol.

Methyl valerenate was synthesized from valerenic acid using diazomethane (chapter 2).

The identity and purity of all test compounds were checked by various spectroscopical and chromatographic techniques (chapters 2 and 3).

Tinctures

Tinctures from subterranean parts of *V. officinalis*, *V. wallichii* and *V. edulis* ssp. *procera* were prepared by percolation of 1 part of ground plant material with 5 parts of ethanol [70%, v/v], according to the German Pharmacopoeia, Anonymous, 1993b). A part of the freshly prepared tinctures was immediately frozen at −20°C until analysed and tested for cytotoxicity. Another part was first stored at room temperature for 2 months before the assays were carried out.

Analytical procedures

Valerenic acid and derivatives, valepotriates and baldrinals, in plant material and in the tinctures, were analysed by HPLC as described in chapter 3. The detection of valerenic acid and derivatives was at 220 nm, of valepotriates at 200 nm (monoene type) or 255 nm (diene type), and of baldrinals at 424 nm (baldrinal, homobaldrinal) or 254 nm (isovaltral). Calibration curves were run with isolated reference compounds.

Cell lines

GLC_4, a human small-cell lung cancer cell line, was derived from a pleural effusion at the Department of Oncology, University Hospital Groningen, The Netherlands (Meijer *et al.*, 1987). The COLO 320 cell line was isolated from a moderately undifferentiated adenocarcinoma of the human colon (Quinn *et al.*, 1979). Both lines are routinely cultured at the Department of Pharmaceutical Biology, Groningen. They are grown in suspension culture, partly floating and partly attached, in RPMI 1640 medium with 25 mM HEPES buffer with L-glutamine (Gibco, Paisly, UK), supplemented with 10% foetal bovine serum (Gibco) plus 200 µg/ml streptomycin and 200 IU/ml penicillin G (Gibco). They were maintained at 37°C in a humidified atmosphere with 5% CO_2. The doubling time was 18-21 h for GLC_4 and 13-15 h for COLO 320. Cells were in the exponential phase of growth at the moment of testing. The viability of the cells used in the experiments exceeded 95% as determined with trypan blue.

Cytotoxicity assay

Cytotoxicity after treatment of the tumour cells with the test compounds or the tinctures was determined using the microculture tetrazolium (MTT) assay. This assay is based on the reduction of the soluble MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] into a blue-purple formazan product, mainly by mitochondrial reductase activity inside living cells (Carmichael *et al.*, 1987). The number of cells was found to be proportional to the extent of formazan production for the cell lines used in this study.

Concentrated stock solutions (200 x) of the test compounds were made in dimethyl sulphoxide (DMSO) and stored at -20°C. The compounds were tested for cytotoxicity in concentrations up to 200 µM. The tinctures were diluted with culture medium; the lowest dilution tested was 1:100. The small amount of DMSO or ethanol present in the wells (maximal 0.5%) was proved not to affect the experiments. Cisplatin (*cis*-dichlorodiammine-platinum(II)), used as a reference cytostatic drug, was obtained from Aldrich (Milwaukee, WI, USA). It was dissolved in water immediately before use.

The cytotoxicity assay was carried out as recently described (Middel *et al.*, 1995; Woerdenbag *et al.*, 1996). Briefly, tumour cells were incubated with a range of concentrations of test compounds in microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) at 37°C in a humidified incubator with 5% CO_2 for a culture period of 4 days. After adding a solution of MTT (Sigma, St. Louis, MO, USA; 5 mg/ml in PBS (phosphate buffered saline; Gibco)), the amount of formazan formed was measured spectroscopically at 515 nm using a Bio-Tek Instruments universal plate reader (Winooski, VT, USA). Cell growth was calculated using the formula: [A_{515} (treated cells) - A_{515} (culture medium)] x 100 / [(A_{515} (control cells) - A_{515} (culture medium)]. The IC_{50} value (concentration of the test compound or dilution of the tincture causing 50% growth inhibition of the tumour cells) was used as a parameter for cytotoxicity.

Statistics

IC_{50} values were calculated using the computer programme GraphPadPrism with nonlinear regression and sigmoidal dose response (variable slope) curve fitting. Standard errors with 95% confidence interval are given.

For comparison of the results of the experiments the paired Student's t-test was used. A p-value <0.05 was considered significant. All cytotoxicity tests were carried out in triplicate.

Results and discussion

In Table 1 the IC_{50} values of characteristic valerian constituents are listed, as determined against GLC_4, a human small-cell lung cancer cell line, and against COLO 320, a human colorectal cancer cell line, using the microculture tetrazolium (MTT) assay; however, the MTT assay does not differentiate between inhibition of cell proliferation and cytotoxicity in terms of cell death. Valepotriates of the diene type (valtrate, isovaltrate and acevaltrate) displayed the highest toxicity, with IC_{50} values slightly higher than those of the reference cytostatic drug cisplatin (GLC_4: 1 µM; COLO 320: 3 µM). The monoene type valepotriates (didrovaltrate and IVHD) were 2- to 3-fold less toxic. Baldrinal, a decomposition product of valtrate and acevaltrate, and homobaldrinal, a decomposition product of isovaltrate, were 10- to 30-fold less toxic than their parent compounds. By contrast, isovaltral, which also originates from isovaltrate, displayed a cytotoxicity even higher than that of the valepotriates. Valerenic acid and its derivatives (acetoxyvalerenic acid, hydroxyvalerenic acid and methyl valerenate), which are only present in *V. officinalis*, possessed a low toxicity with IC_{50} values between 100 µM and 200 µM. COLO 320 was significantly ($p<0.05$) more resistant to the valepotriates and baldrinals than GLC_4. For valerenic acid

Table 1. Cytotoxicity of valerian constituents [IC_{50} values (µM) ± standard error (95% confidence interval); n = 3]

	GLC_4	COLO 320
Valepotriates		
- diene type		
Valtrate	1.4 ± 0.1	3.0 ± 0.3
Isovaltrate	2.5 ± 0.1	5.4 ± 0.5
Acevaltrate	1.3 ± 0.1	3.6 ± 0.2
- monoene type		
Didrovaltrate	8.9 ± 0.4	15.2 ± 0.8
Isovaleroxyhydroxydidrovaltrate	2.4 ± 0.2	7.2 ± 0.2
Baldrinals		
Baldrinal	53 ± 2	111 ± 5
Homobaldrinal	31 ± 2	57 ±10
Isovaltral	0.4 ± 0.1	1.2 ± 0.1
Valerenic acid and derivatives		
Valerenic acid	127 ± 9	124 ± 8
Acetoxyvalerenic acid	111 ± 8	124 ± 7
Hydroxyvalerenic acid	123 ± 4	165 ± 3
Methyl valerenate	115 ± 2	183 ± 14

and its derivatives this difference in sensitivity of the cell lines was less pronounced or absent.

For several essential oil components of valerian, including valeranone, kessoglycyl monoacetate, kessoglycyl diacetate, cryptofauronol, patchouli alcohol and maaliol, cytotoxic effects comparable to valerenic acid and its derivatives were found (data not given).

In Table 2 a survey is given of the contents of characteristic valerian constituents in the plant material and in freshly prepared and stored tinctures. Extraction of the plant material (10 g) with methanol (3 x 30 ml) was exhaustive with respect to the compounds listed. The tinctures, prepared with 70% ethanol, contained considerably less of the constituents. Compared with the methanolic extract, the recovery of valerenic acid and acetoxyvalerenic acid in the tincture of *V. officinalis* was about 50%. Hydroxyvalerenic acid was not extracted at all with 70% ethanol. The recovery of valepotriates in the fresh tinctures was between 50% and 80%. The fresh tinctures of *V. wallichii* and *V. edulis* ssp. *procera* already contained more baldrinals than the crude plant material, indicating that the decomposition of valepotriates already took place during the percolation procedure.

Following a storage period of 2 months, most of the valepotriates originally present in the tinctures had decomposed (Table 2). This was reflected in a significant reduction of the cytotoxic effect of the stored tinctures as compared with the corresponding fresh ones. The decomposition of the valepotriates did, however, not result in a concomitant rise of the baldrinal content. Isovaltral was not detected at all in the tinctures.

In Table 3 the dilutions of the tinctures causing 50% effect in the MTT assay are given, as well as the ratio between the cytotoxicity of the fresh and stored tinctures. The higher this ratio, the more decomposition of valepotriates had occurred upon storage. The fresh tincture of *V. edulis* ssp. *procera*, having the highest valepotriate content, had the highest cytotoxicity; the fresh tincture of *V. officinalis*, with a low valepotriate content, was least toxic. After storage, the largest reduction in cytotoxicity was found for the tinctures of *V. officinalis* and *V. edulis* ssp. *procera*.

In the present study the cytotoxicity of the characteristic valerian constituents is compared in one test system, which might be helpful in evaluating

Table 2. Contents of valepotriates, baldrinals, and valerenic acids in subterranean parts of *Valeriana officinalis*, *V. wallichii* and *V. edulis* ssp. *procera* (in mg/g plant material), and in freshly prepared and stored tinctures thereof (in mg/100 ml tincture)

	Valeriana officinalis			*Valeriana wallichii*			*Valeriana edulis*		
	SP[a]	FT[b]	ST[c]	SP[a]	FT[b]	ST[c]	SP[a]	FT[b]	ST[c]
Valepotriates									
Valtrate	4.4	44.0	2.4	4.1	54.8	19.9	31.9	549.5	3.5
Isovaltrate	46.9	503.0	20.5	2.9	39.6	4.3	152.2	2625.0	6.0
Didrovaltrate	n.d.[d]	n.d.	n.d.	4.8	68.2	68.8	8.6	109.0	103.0
Baldrinals									
Baldrinal	n.d.	n.d.	n.d.	0.06	0.73	0.63	0.04	0.9	0.6
Homobaldrinal	n.d.	n.d.	n.d.	n.d.	0.83	2.86	n.d.	3.2	10.9
Valerenic acids									
Valerenic acid	4.8	52.9	52.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Acetoxyvalerenic acid	3.0	29.2	31.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Hydroxyvalerenic acid	3.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

[a]Subterranean parts; [b]freshly prepared tincture; [c]stored tincture; [d]not detected.

Table 3. Cytotoxicity of valerian tinctures, freshly prepared from subterranean parts of *Valeriana officinalis*, *V. wallichii* and *V. edulis* ssp. *procera*, and after a storage period of 2 months [IC_{50} values ± standard error (95% confidence interval); n = 3; in terms of the dilution causing 50% effect in the MTT assay]

GLC_4			
	Fresh (F)	Stored (S)	ratio F:S
V. officinalis	1221 ± 41	329 ± 8	3.7
V. wallichii	3501 ± 338	2663 ± 83	1.3
V. edulis	9364 ± 329	1983 ± 76	4.7
COLO 320			
	Fresh (F)	Stored (S)	ratio F:S
V. officinalis	582 ± 15	152 ± 4	3.8
V. wallichii	1425 ± 61	1240 ± 65	1.1
V. edulis	4662 ± 263	1132 ± 77	4.1

the safety of phytomedicines containing these compounds. For the production of solid oral dosage forms (capsules, film-coated tablets), *V. officinalis*, *V. wallichii* and *V. edulis* ssp. *procera* are used. From *V. officinalis* also tinctures and teas are prepared. Phytomedicines that are standardized on valepotriates are mostly prepared from *V. wallichii* and *V. edulis* ssp. *procera*, because these species are relatively rich in valepotriates (Wichtl 1989, Bos *et al.*, 1992).

The diene valepotriates were significantly more toxic than the monoene valepotriates (Table 1). This can be explained in relation to their reactivity: diene valepotriates decompose upon storage, whereas the monoene valepotriates remain stable (Table 2). Despite the decomposition of the diene valepotriates, as seen in the stored tinctures, the amounts of baldrinals formed are low. It is suggested that they react further to yield yet unknown products (chapters 1 and 3).

Comparing the data from the Tables 2 and 3, a clear relationship is seen between the valepotriate content of a tincture and its cytotoxicity. The amount of valepotriates present in the dilution of a tincture causing 50% effect in the MTT assay, was in all cases between 0.3 and 0.7 mg/ml, as calculated from the data in these tables. The IC_{50} values of valtrate, isovaltrate and didrovaltrate were 0.6, 1.0 and 3.4 µg/ml, respectively. This means that most of the cytotoxic effect of the valerian tinctures can be attributed to the presence of valepotriates.

The valepotriates are alkylating agents, and the epoxy group is responsible for this property. They are supposed to display cytotoxic effects by interacting with thiol-containing enzymes. The addition of compounds with free

thiol groups (e.g. cysteine and glutathione) has been found to counteract the cytotoxicity of valepotriates in cultured hepatoma cells (Keochanthala-Bounthanh *et al.*, 1990). This indicates that intracellular biological nucleophiles are able to protect the cell against the cytotoxic effect of valepotriates, by covalent and non-covalent binding. COLO 320 has higher cellular gluthathione levels than GLC_4, and therefore possesses a higher capacity to detoxify alkylating agents (Woerdenbag *et al.*, 1994). This may explain the difference in sensitivity between the cell lines for the valepotriates as well as for the reference cytostatic drug cisplatin. In addition, valepotriates have been found to inhibit the synthesis of DNA and of proteins (Keochanthala-Bounthanh *et al.*, 1993).

It is, however, unclear to what extent the cytotoxic effects of valepotriates and, to a lesser extent, of baldrinals are relevant for humans after ingestion of valerian preparations. Nevertheless, more and more preference is given to valerian preparations that are devoid of these potentially hazardous constituents (De Smet and Vulto, 1988; Hänsel, 1992). A tincture of *V. officinalis* stored for at least 1-2 months may therefore be preferred over valepotriate-rich phytomedicines.

Final remarks and perspectives

Phytomedicines are complex mixtures of substances, and crude plant material used for their preparations may vary considerably in its content of active ingredients. Additional alterations of plant constituents may be induced by the manufacturing process of phytomedicines. Therefore the demands of the manufacturer can only be met by a comprehensive quality control. This control should include the crude plant material, the manufacturing process as well as the standardized final product. It is recommended, also with respect to valerian based phytomedicines, that not only the identity of the plant material and the extraction procedure should be documented, but also the amount of the components present in the preparations on which they may eventually be standardized. The HPLC method described in this thesis is suitable for these purposes and can be used as a further starting point for an improved monograph on valerian in the next edition of the European Pharmacopoeia.

In the near future, registration of phytomedicines will become obligatory in the countries of the European Union. Reliable data on the chemistry, pharmacology, toxicology and clinical effectiveness of valerian will thus be needed. Despite the broad use of valerian based phytomedicines, especially well-conducted clinical studies are scarce.

In addition, selection and breeding programmes will also be necessary in the future, in order to guarantee a continuous supply of plant material of high quality. For the production of valerian, seeds with a high germination capacity are necessary. Of further importance is a large production (yield; kg/ha) of subterranean parts. High-quality valerian should have an essential oil content of 1.0-1.5%, a valerenic acid content of $\geq$ 0.5% and an extractable matter of $\geq$ 30%. For harvesting purposes, the plant material should have a small rhizome and a large amount of unbranched roots, which can easily be worked up. From an environmental point of view, seeds should be selected from plants which are more or less resistant against several types of mildew, and against *Phoma* and *Verticillium dahliae*. Also plant louse and thrips often cause damage to the plant material. It is important to use as little as possible pesticides. Heavy metals as lead and cadmium taken up from polluted environments are expected to give a rise of difficulties in the near future.

Summary

In The Netherlands and in other European countries the subterranean parts of three valerian species, namely *Valeriana officinalis* L. *s.l.*, *V. wallichii* DC. and *V. edulis* Nutt. ex Torr. & Gray ssp. *procera* (H.B.K.) F.G. Meyer, are used as mild sedatives, but they show large differences with regard to their constituents. Consequently, phytomedicines prepared from these species show different chemical compositions. It is still not fully clear which constituents are responsible for the sedative action, but the valepotriates as well as valerenic acid and derivatives thereof are generally considered to contribute to it. In addition, the essential oil may play a role in the biological activity of valerian. Quality assurance and quality control of the crude drug and its preparations should therefore be based on these groups of secondary metabolites. The aim of the studies described in this thesis was to characterize valerian species and valerian preparations with respect to their contents of valerenic acid and its derivatives, valepotriates, and essential oil components.

In chapter 1, botanical, chemical and analytical aspects related to the medicinally used valerian species are extensively reviewed as well as their use, pharmacology and toxicology.

Phytochemical investigations of *V. officinalis* roots and rhizomes carried out in our institute during the last 25 years yielded several compounds, including some new ones. The results of some of these investigations are described in chapter 2. From the essential oil and a methylene chloride extract from the subterranean parts of the plant a number of cyclopentane sesquiterpenoids have been isolated and identified. Valerenic acid, hydroxyvalerenic acid, acetoxyvalerenic acid and valerenal were already known constituents of this species. In addition to these compounds, valerenol, a number of valerenyl esters, faurinone, (-)-pacifigorgiol and methyl valerenate were isolated and identified in our laboratory.

In chapter 3, a high-performance liquid chromatography (HPLC) method with diode array detection is described, by which valerenic acid, and its derivatives hydroxyvalerenic acid and acetoxyvalerenic acid, as well as the valepotriates and their decomposition products baldrinal and homobaldrinal can be separated and measured in one run. Using this procedure, roots and rhizomes of *V. officinalis* were analysed. Furthermore, the influence of different ethanol-water mixtures, used as extraction liquid, on the composition of extracts of *V.*

officinalis is reported. At an ethanol concentration of 30%, valerenic acid and its derivatives started to be extracted, and at ethanol concentrations > 50% the amounts of valerenic acid and its derivatives were more or less constant. Valepotriates were extracted only with ethanol concentrations > 70%.

The analytical procedure was also applied to a number of valerian based phytomedicines available on the Dutch market. Apart from the variation in crude drugs used, we found discrepancies with respect to standardization and to the maximum daily dosage. In most cases, only capsules had been standardized with respect to valepotriates, whilst tinctures and film-coated tablets were often not standardized. Remarkable differences were found with respect to the maximum daily dose mentioned, varying from 45 to 180 drops (2-8 ml), from 6 to 12 film-coated tablets, and from 3 (27.5 mg valepotriate/capsule) to 8 capsules (48 mg valepotriate/capsule). The decomposition products baldrinal and homobaldrinal could only be detected in very small amounts in some of the capsules.

In order to study the stability of the valepotriates and the formation of their decomposition products, each day samples of freshly prepared tinctures were analysed after storage at 4, 20 and 36°C for up to one month. In a solution with 70% ethanol the concentration of valepotriates decreased to 30% of the original level after a two-weeks storage period at 20°C, and almost nothing had remained after 3-4 weeks, while the baldrinal content rapidly increased from 5% after 2 weeks to 85% after 3-4 weeks. Upon storage at 36°C the valepotriate content of the tincture decreased even more rapidly, but baldrinals were not detected. A large part of the baldrinals may react with other, yet unknown, constituents present in a tincture and this may explain the lack of baldrinals in stored valerian tinctures.

In the chapters 4, 5 and 6, studies on the essential oil composition of three valerian species are described. The investigations were performed by GC and GC-MS analysis.

Chapter 4 deals with the volatile constituents from roots and rhizomes of *Valeriana officinalis* L. *s.l.* and several closely related *Valeriana* taxa. Most of the seeds came from different botanical gardens in Europe, and plants were grown in an experimental field in The Netherlands. In addition, commercially available plant material of Dutch origin was investigated.

V. officinalis is a collective species. Four subspecies were included in our study. The roots and rhizomes of 16 *V. officinalis* ssp. *officinalis* samples yielded 0.22-1.55% of essential oil. Oil components found in all samples were bornyl acetate (2-36%), myrtenyl acetate (trace-9%), nojigiku acetate (trace-

0.8%), valerenic acid (0.3-3%), and a yet unidentified tertiary sesquiterpene alcohol with a retention index (RI) of 1597. The yield for two samples of *V. officinalis* ssp. *collina* was 0.78% and 0.85% of essential oil. The main components were bornyl acetate (22-24%), myrtenyl acetate (6-8%), camphene (4-7%), kessane (3-6%), β-eudesmol (4-5%) and a tertiary sesquiterpene alcohol (RI = 1622) amounting to 20%, which was present in one sample only. For seven samples of *V. officinalis* ssp. *sambucifolia* the yield of oil was 0.19-1.6%, with main components bornyl acetate (4-25%) and valerianol (3-34%). The only sample of *V. repens* yielded 0.34% oil, with bornyl acetate (13%), valerianol (19%) and kessane (8%) as the main components.

Among the other (sub)species investigated in this study, the oil of *V. celtica* ssp. *norica* contained bornyl acetate (22%) and patchouli alcohol (5%). Patchouli alcohol was also present in the oil of the two samples of *V. phu*, together with γ-patchoulene, patchoulyl acetate and a valerenal isomer (15-19%). Valeranone (16%) was the main constituent of the oil of *V. exaltata*. Bornyl acetate and valerianol were also present in the essential oils of the other related taxa studied.

In addition, the subterranean parts were analysed by HPLC for valerenic acid and valepotriates. Although valerenic acid is recognized as a characteristic compound of *V. officinalis*, we also found this sesquiterpenoid in *Valeriana* taxa that are not considered as members of the collective species. It is concluded that either valerenic acid is not exclusive for the collective species *V. officinalis*, or the collective species should be extended with some other subspecies. In addition, the presence of valepotriates is reported in species that were not known to contain these compounds so far.

In chapter 5, the volatile constituents isolated from roots and rhizomes of *Valeriana wallichii* DC. were investigated. The plant material was obtained from different sources, namely from a local market in Kathmandu (Nepal), from plants grown in The Netherlands and in Germany, and from commercially available material from different German importers. The various roots and rhizomes yielded between 0.09% and 1.3% essential oil. The main constituents of the oils from the plants grown in Europe were an unidentified sesquiterpene hydrocarbon, α-santalene, *ar*-curcumene and xanthorrhizol. In the oils from the Nepalese material and from the commercially available roots, except in one sample, patchouli alcohol was the main component.

Chapter 6 deals with the volatile constituents from roots and rhizomes of *Valeriana javanica* Blume, grown in Indonesia. This material yielded 0.36%

essential oil. Main constituents of the oil were α-kessyl acetate (36%), α-cedrene (15%), patchouli alcohol (19%), kessane (7%), valeranone (3%) and bornyl acetate (3%). Root extracts were analysed by HPLC for valepotriates, and valerenic acid and its derivatives. The valepotriates included valtrate (2.2%), isovaltrate (0.6%) and acevaltrate (0.1%). Valerenic acid and derivatives were not detected. From a phytochemical point of view, *V. javanica* is not comparable with the *Valeriana* species used in Western countries. However, some similarity exists with the Japanese species *V. fauriei* Briq.

In chapter 7, the seasonal variation in the contents of the essential oil, valerenic acid and derivatives, and valepotriates in *V. officinalis* roots and rhizomes, and the selection of plants suitable for phytomedicines are described. During the seasons 1989-1993, subterranean parts of *V. officinalis* plants were investigated for their contents of essential oil, valerenic acid and derivatives, and valepotriates. Harvesting of the subterranean parts was started in August of the year in which the seeds were sown, and continued until the last week of April of the subsequent year. Despite usual variations from year to year, the maximum contents of essential oil in the subterranean parts of *V. officinalis* were found in September, ranging from 1.2% to 2.1%. Over the vegetation periods investigated, the composition of the oil remained more or less constant. Valerenic acid and its derivatives, and the valepotriates reached their maximum amounts in February-March, with contents of 0.7-0.9% and 1.1-1.4%, respectively.

During the period 1989-1993, five *V. officinalis* strains were investigated for their contents of essential oil, valerenic acid and derivatives, and valepotriates in order to select plants suitable for phytomedicines. The selection procedures described, eventually (in 1993) yielded plant material with a satisfactory content of essential oil (0.9%) combined with a high content of valerenic acid and derivatives (0.5%) which could be harvested in September of the year of sowing.

Characteristic constituents of three *Valeriana* species were tested for cytotoxicity against a human small-cell lung cancer cell line (GLC_4), and against COLO 320, a human colorectal cancer cell line, using the microculture tetrazolium (MTT) assay as described in chapter 8. Valepotriates of the diene type displayed the highest cytotoxicity, with IC_{50} values of 1-6 μM. The monoene type valepotriates were 2- to 3-fold less toxic. The decomposition products of valepotriates, baldrinal and homobaldrinal, were 10- to 30-fold less toxic than their parent compounds but isovaltral had a higher cytotoxicity than its parent compound isovaltrate. Valerenic acid and its derivatives (acetoxyvalerenic acid, hydroxyvalerenic acid and methyl valerenate) showed a low toxicity with

IC_{50} values between 100 μM and 200 μM. Freshly prepared and stored tinctures, prepared from roots and rhizomes of the three valerian species, namely *V. officinalis s.l.*, *V. wallichii* and *V. edulis* ssp. *procera*, were also tested for cytotoxicity. There was a clear relationship between the valepotriate contents of the freshly prepared tinctures and their toxicity. Upon storage, valepotriates decomposed, which was reflected in a significant reduction of the cytotoxic effect.

Samenvatting

In Nederland en in andere Europese landen worden de ondergrondse delen (wortel en wortelstokken) van drie valeriaansoorten, te weten *Valeriana officinalis* L. *s.l.*, *V. wallichii* DC. en *V. edulis* Nutt. ex Torr. & Gray ssp. *procera* (H.B.K.) F.G. Meyer, gebruikt als uitgangsmateriaal voor milde sedativa, hoewel zij kwalitatief en kwantitatief verschillen wat betreft hun inhoudsstoffen. Fytofarmaca die bereid zijn uit deze valeriaansoorten, verschillen hierdoor ook in samenstelling. Tot op heden is er niet eenduidig een stof of stofgroep aan te wijzen die voor de werking verantwoordelijk is, maar men denkt dat de valepotriaten alsook valereenzuur en valereenzuurderivaten hiertoe zeker bijdragen. Ook de vluchtige olie kan een rol spelen bij de biologische activiteit van valeriaan. Kwaliteitswaarborging en kwaliteitscontrole van het uitgangsmateriaal en van daaruit bereide preparaten moeten daarom gebaseerd zijn op deze groepen secundaire metabolieten. Het doel van het in dit proefschrift beschreven onderzoek was het karakteriseren van valeriaansoorten en valeriaanpreparaten met betrekking tot hun gehalte aan valereenzuur en valereenzuurderivaten, valepotriaten en vluchtige-oliebestanddelen.

In hoofdstuk 1 wordt een uitgebreid overzicht gegeven van de botanische, chemische en analytische aspecten van de valeriaansoorten die voor fytofarmaca worden gebruikt. Tevens worden hun gebruik, farmacologie en toxiciteit besproken.

Fytochemisch onderzoek van de wortels en wortelstokken van *V. officinalis* dat de afgelopen 25 jaar in ons instituut is uitgevoerd, heeft een aantal geïsoleerde - soms nieuwe - verbindingen opgeleverd. De resultaten van een aantal van deze studies zijn beschreven in hoofdstuk 2. Uit de vluchtige olie en uit een dichloormethaanextract uit de ondergrondse delen van deze plant is een aantal cyclopentaan-sesquiterpenen geïsoleerd en geïdentificeerd. Valereenzuur, hydroxyvalereenzuur, acetoxyvalereenzuur en valerenal waren reeds bekende verbindingen uit *V. officinalis*. In aansluiting hierop zijn valerenol, een aantal valerenylesters, faurinon, (-)-pacifigorgiol en methylvalerenaat in ons laboratorium geïsoleerd en geïdentificeerd.

In hoofdstuk 3 wordt een hoge-druk-vloeistofchromatografische methode (high-performance liquid chromatography, HPLC) met diode-array-detectie beschreven, waarmee zowel valereenzuur, hydroxyvalereenzuur en

acetoxyvalereenzuur alsook de valepotriaten en hun ontledingsproducten baldrinal en homobaldrinal gescheiden en gemeten kunnen worden in één enkele analyse. Gebruikmakend van deze methode werden de ondergrondse delen van *V. officinalis* geanalyseerd. Tevens werd de invloed van verschillende ethanol-watermengsels die als extractievloeistof werden gebruikt, op de samenstelling van de *V. officinalis*-extracten onderzocht. Eerst bij een ethanolgehalte ≥ 30% werden valereenzuur en zijn derivaten geëxtraheerd, en bij een ethanolgehalte > 50% bleef de geëxtraheerde hoeveelheid valereenzuur en derivaten nagenoeg constant. Valepotriaten werden slechts geëxtraheerd bij een ethanolgehalte > 70%.

De analysemethode is ook toegepast voor de analyse van een aantal in Nederland verkrijgbare uit valeriaan bereide fytofarmaca. Er werden grote verschillen gevonden zowel in de samenstelling als in de maximale dagelijkse dosis, zoals aangegeven door de fabrikanten. In de meeste gevallen werd alléén voor capsules een hoeveelheid valepotriaten opgegeven. Bij zowel de tincturen als bij de dragees werd vaak alleen de hoeveelheid extract vermeld en in een enkel geval ook het uitgangsmateriaal. De aangegeven maximale dagelijkse dosis varieerde van 45 tot 180 druppels tinctuur (2-8 ml), van 6 tot 12 dragees en van 3 (27,5 mg valepotriaat/capsule) tot 8 capsules (48 mg valepotriaat/capsule). De ontledingsproducten baldrinal en homobaldrinal konden alleen in zeer geringe hoeveelheden in enkele capsules worden aangetoond.

Om de stabiliteit van de valepotriaten en de vorming van hun ontledingsproducten te bestuderen werden vers bereide tincturen gedurende 1 maand bewaard bij 4, 20 en 36°C en werden er dagelijks monsters genomen en geanalyseerd. In een 70% ethanol-oplossing daalde, na twee weken bewaren bij een temperatuur van 20°C, het valepotriaatgehalte naar 30% van het oorspronkelijke niveau, terwijl na 3-4 weken helemaal geen valepotriaten meer konden worden aangetoond. Tegelijkertijd nam de hoeveelheid baldrinalen snel toe, van 5% na 2 weken tot 85% na 3-4 weken. In een valeriaantinctuur, bewaard bij 36°C, ontleedden de valepotriaten zeer snel, maar baldrinalen waren niet aantoonbaar. Een groot deel van de baldrinalen reageert waarschijnlijk met andere, nog onbekende, stoffen die in de tinctuur aanwezig zijn en dit verklaart voor een deel ook het ontbreken van baldrinalen in oudere valeriaantincturen op de Nederlandse markt.

In de hoofdstukken 4, 5 en 6 wordt de vluchtige-oliesamenstelling van drie verschillende valeriaansoorten behandeld. Het onderzoek werd uitgevoerd

met behulp van gaschromatografie (GC) en gaschromatografie gekoppeld met massaspectrometrie (GC-MS).

Hoofdstuk 4 betreft de vluchtige componenten uit de wortel en de wortelstok van *V. officinalis* L. *s.l.* en uit een aantal verwante taxa. Het zaadmateriaal, dat afkomstig was van verschillende botanische tuinen in Europa, werd uitgezet op een proefveld in Nederland. Tegelijkertijd werd ook de vluchtige olie uit commercieel in Nederland verkrijgbaar plantenmateriaal onderzocht.

V. officinalis is de verzamelnaam voor een aantal ondersoorten. Vier van deze ondersoorten werden onderzocht. Het vluchtige-oliegehalte van de wortels en wortelstokken van 16 monsters van *V. officinalis* ssp. *officinalis* lag tussen 0,22% en 1,55%. Componenten die in elk van deze oliën aangetoond werden, waren bornylacetaat (2-36%), myrtenylacetaat (spoor-9%), nojigiku-acetaat (spoor-0,8%), valereenzuur (0,3-3%) en een tot op dit moment niet geïdentificeerde sesquiterpeenalcohol met een retentie-index (RI) van 1597. Het vluchtige-oliegehalte van twee monsters van *V. officinalis* ssp. *collina* bedroeg 0,78% en 0,85%. De hoofdcomponenten waren bornylacetaat (22-24%), myrtenylacetaat (6-8%), kamfeen (4-7%), kessaan (3-6%), β-eudesmol (4-5%) en - in één van beide monsters - een tertiaire sesquiterpeenalcohol (RI = 1622) in een percentage van 20%. Voor zeven monsters van *V. officinalis* ssp. *sambucifolia* lag het vluchtige olie-gehalte tussen 0,19% en 1,6%, met als hoofdcomponenten bornylacetaat (4-25%) en valerianol (3-34%). Het enige onderzochte monster van *V. repens* had een vluchtige-oliegehalte van 0,34%, met bornylacetaat (13%), valerianol (19%) en kessaan (8%) als de belangrijkste bestanddelen.

Van andere (onder)soorten die onderzocht werden, bevatte de vluchtige olie van *V. celtica* ssp. *norica* 22% bornylacetaat en 5% patchouli-alcohol. Patchouli-alcohol was ook aanwezig in de vluchtige olie van de twee monsters van *V. phu*, samen met γ-patchouleen, patchoulylacetaat en een valerenal-isomeer met een gehalte tussen 15% en 19%. Valeranon was met een percentage van 16% de hoofdcomponent van de vluchtige olie van *V. exaltata*. Bornylacetaat en valerianol waren ook aanwezig in de vluchtige oliën van de overige onderzochte verwante taxa.

De ondergrondse delen werden voorts onderzocht op de aanwezigheid van valereenzuur, zijn derivaten en valepotriaten. Hoewel valereenzuur geldt als karakteristieke inhoudsstof voor *V. officinalis*, hebben we deze sesquiterpeen ook aangetoond in een aantal taxa die niet tot *V. officinalis*

worden gerekend. Uit deze gegevens kan worden geconcludeerd, dat valereenzuur niet uitsluitend in taxa voorkomt die tot *V. officinalis* worden gerekend, of dat deze soort moet worden uitgebreid met een aantal andere ondersoorten. Ook werden valepotriaten aangetoond in soorten waarvan niet eerder bekend was dat ze deze verbindingen bevatten.

In hoofdstuk 5 wordt de analyse van vluchtige olie uit de ondergrondse delen van *V. wallichii* DC. beschreven. Het plantenmateriaal was afkomstig van verschillende herkomsten. Gedroogd materiaal werd gekocht op een markt in Kathmandu (Nepal), vers materiaal was afkomstig van planten die in Nederland en Duitsland werden gekweekt, en er was commercieel verkrijgbaar materiaal van enkele Duitse importeurs. De verschillende monsters leverden tussen de 0,09% en 1,3% vluchtige olie op. De hoofdbestanddelen van de vluchtige olie van het uit Europa afkomstige materiaal waren een niet geïdentificeerde sesquiterpeenkoolwaterstof, α-santaleen, *ar*-curcumeen en xanthorrhizol. Patchouli-alcohol was het hoofdbestanddeel van de olie van zowel het Nepalese als van het commercieel verkrijgbare materiaal, met uitzondering van één monster.

Hoofdstuk 6 betreft de samenstelling van de vluchtige olie uit de ondergrondse delen van *V. javanica* Blume, afkomstig uit Indonesië. Het vluchtige-oliegehalte bedroeg 0,36%. Hoofdcomponenten van de vluchtige olie waren α-kessylacetaat (36%), α-cedreen (15%), patchouli-alcohol (19%), kessaan (7%), valeranon (3%) en bornylacetaat (3%). Het wortelmateriaal werd door middel van HPLC ook geanalyseerd op de aanwezigheid van valepotriaten, en van valereenzuur en zijn derivaten. De volgende, reeds bekende, valepotriaten werden aangetoond: valtraat (2,2%), isovaltraat (0,6%) en acevaltrate (0,1%). Valereenzuur en zijn derivaten konden niet worden aangetoond. In fytochemisch opzicht komt *V. javanica* niet overeen met de in de Europese landen gebruikte valeriaansoorten. Wel bestaat er enige overeenkomst met de in Japan voorkomende soort *V. fauriei* Briq.

In hoofdstuk 7 wordt de seizoenvariatie in het vluchtige-oliegehalte, in het gehalte aan valereenzuur en valereenzuurderivaten en in het valepotriaatgehalte van de ondergrondse delen van *V. officinalis* beschreven. Ook wordt de mogelijkheid van selectie van planten, die geschikt zijn voor de bereiding van fytofarmaca, beschreven. Gedurende de seizoenen 1989-1993 werden ondergrondse delen van *V. officinalis*-planten onderzocht op hun gehaltes aan vluchtige olie, aan valereenzuur en derivaten, en aan valepotriaten. De monstername vond plaats tussen augustus van het jaar van uitzaai tot en met de

laatste week van april van het daarop volgende jaar. Afgezien van de gebruikelijke variatie van jaar tot jaar, werd de maximale hoeveelheid vluchtige olie gevonden in september. Deze varieerde van 1,2% tot 2,1%. Gedurende de onderzochte vegetatieperiodes bleef de samenstelling van de vluchtige olie nagenoeg constant. Het maximale gehalte aan valereenzuur en derivaten en aan valepotriaten werd gevonden in februari-maart; het bedroeg respectievelijk 0,7-0,9% en 1,1-1,4%.

Gedurende de periode 1989-1993 werd materiaal van vijf verschillende *V. officinalis*-lijnen onderzocht op vluchtige-oliegehalte, gehalte aan valereenzuur en derivaten, en valepotriaatgehalte, met als doel planten te selecteren die geschikt zijn voor het bereiden van fytofarmaca. De beschreven selectieprocedure leverde uiteindelijk (in 1993) plantenmateriaal op met een acceptabel vluchtige-oliegehalte (0,9%), gecombineerd met een hoog gehalte aan valereenzuur en derivaten (0,5%), en bovendien kon het materiaal worden geoogst in september van het jaar van uitzaaien.

In hoofdstuk 8 wordt behandeld hoe karakteristieke verbindingen uit wortel en wortelstokken van valeriaansoorten werden getest op cytotoxiciteit tegen een menselijke kleincellige longkankercellijn (GLC_4) en tegen COLO 320, een menselijke dikke-darmkankercellijn, gebruikmakend van de 'microculture tetrazolium (MTT) assay'. Valepotriaten van het dieen-type bezaten de hoogste cytotoxiciteit, met IC_{50}-waarden van 1-6 μM. De valepotriaten van het monoeen-type waren 2- tot 3-maal minder toxisch. De ontledingproducten van de valepotriaten, baldrinal en homobaldrinal, waren 10- tot 30-maal minder toxisch dan hun uitgangsstoffen, maar isovaltral had een hogere toxiciteit dan zijn uitgangsstof isovaltraat. Valereenzuur en zijn derivaten (acetoxyvalereenzuur, hydroxyvalereenzuur en methylvalerenaat) bezaten een lage toxiciteit, met IC_{50}-waarden tussen 100 μM en 200 μM. Ook werden vers bereide en enige tijd bewaarde tincturen van de ondergrondse delen van *V. officinalis s.l., V. wallichii* en *V. edulis* ssp. *procera* getest op hun cytotoxiciteit, en werd daarin het valepotriaatgehalte gemeten. Er bestond een duidelijke relatie tussen het valepotriaatgehalte van de tincturen en hun cytoxiciteit. De vers bereide tinctuur van *V. edulis* met een hoog valepotriaatgehalte bezat een duidelijk hogere toxiciteit dan de beide andere tincturen met een lager valepotriaatgehalte. Een duidelijke afname van de cytotoxiciteit was waarneembaar bij bewaarde tincturen, waarin de valepotriaten ontleed waren. Gezien de hogere cytotoxiciteit van valepotriaat-bevattende tincturen is het raadzaam de voorkeur te geven aan tincturen bereid

uit *V. officinalis*. Zij bevatten valereenzuur en derivaten ervan die veel minder cytotoxisch zijn en voorts gedurende lange tijd stabiel blijven. Bovendien kunnen tincturen hierop worden gestandaardiseerd.

Ondanks de uitgebreide toepassing van valeriaanpreparaten zijn er slechts enkele goed gedocumenteerde klinische studies bekend.

Om ook in de toekomst verzekerd te zijn van plantenmateriaal dat voldoet aan de eisen die gesteld worden in de Europese Farmacopee, speelt een aantal zaken een rol van betekenis. Veredeling via zaad is en blijft belangrijk met als doel een productie van zaad met hoge kiemkracht, en plantenmateriaal met een vluchtige-oliegehalte van 1,0-1,5%, een extractgehalte van minimaal 30% en een valereenzuurgehalte van 0,5%.

Het gebruik van herbiciden zal zo laag mogelijk moeten worden gehouden. Van zware metalen zoals lood en cadmium is bekend dat ze, afhankelijk van de grondsoort, snel in de wortels worden opgenomen. Dit kan, in het kader van nieuwe kwaliteitseisen, in de toekomst problemen veroorzaken. Vanuit milieu-overwegingen, om het gebruik van bestrijdingsmiddelen zoveel mogelijk te beperken, is een bepaalde mate van resistentie ten opzichte van echte en valse meeldauw, bladluis, *Phoma* en *Verticillium dahliae* belangrijk. In verband met het oogsten en verwerken van het wortelmateriaal zal de selectie gericht moeten zijn op planten met een kleine wortelstok en een grote hoeveelheid wortels, hoewel bekend is dat het vluchtige-oliegehalte in de wortelstok hoger is dan in het wortelmateriaal.

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Nawoord

Het onderzoek dat ik in dit proefschrift heb beschreven, is voor een deel het resultaat van een goede samenwerking tussen de werkgroep Farmaceutische Biologie en de Verenigde Nederlandse Kruidencoöperatie in Elburg. Nu het proefschrift klaar is, wil ik iedereen die op zijn of haar manier een bijdrage heeft geleverd heel hartelijk bedanken.

Mijn promotores, Prof. Dr. H.V. Wikström en Prof. Dr. J.J.C. Scheffer ben ik dank verschuldigd voor hun goede begeleiding, hun bereidheid als promotor op te treden, en voor het kritisch lezen van het manuscript. Hans Scheffer ben ik bovendien nog dankbaar voor het zeer consciëntieus nakijken van het manuscript waarmee vele uren gemoeid moeten zijn geweest.

Dr. Herman Woerdenbag was, na het overlijden van Prof. Dr. Th.M. Malingré, het eerste aanspreekpunt. Herman, ik heb je het afgelopen jaar tevens leren waarderen als kamergenoot, hoewel klassieke muziek, en met name opera, niet tot je favoriete genre behoorde. Vooral voor je geduld, je substantiële bijdrage aan het geheel en de praktische bijdrage aan het laatste hoofdstuk ben ik je veel dank verschuldigd.

Dr. Henk Hendriks ben ik zeer erkentelijk voor zijn kritische inbreng bij onze gezamenlijke publicaties en het becommentariëren van het manuscript.

Zonder het enthousiasme van Freeke van Putten, die ondanks de onvermijdelijke stofwolken en de daaropvolgende niesbuien het grootste deel van de monstervoorbereiding verzorgde, was het aantal verkregen resultaten niet zo groot geweest.

Ik ben de werkgroep Farmaceutische Biologie erkentelijk, in eerste instantie wijlen Prof. Dr. Th.M. Malingré, voor de toestemming om een promotie-onderzoek te mogen starten, en in een later stadium Dr. Jan Zwaving en Dr. Niesko Pras, beiden als werkgroepleider a.i., voor de mogelijkheid en de vrijheid die zij mij gaven om het onderzoek te kunnen voortzetten en afronden.

Niet te vergeten, mijn dank geldt ook alle ondersteunende diensten van het Universitair Centrum voor Farmacie, met name Dr. Andries Bruins en Margot Jeronimus-Stratingh van de massaspectrometrieafdeling, die er steeds weer in slaagden de Finnigan bedrijfsklaar te houden.

Zonder plantenmateriaal was dit onderzoek niet mogelijk geweest. Jan Visser ben ik zeer erkentelijk voor het verzamelen van de verschillende zaadmonsters en het opkweken van het jonge plantenmateriaal.

Voor het gebruik van de proeftuin in Elburg ben ik het Bestuur van de Verenigde Nederlandse Kruidencoöperatie u.a. en vooral de directeur A.J.

Ottens veel dank verschuldigd. Dankzij deze faciliteit was er altijd voldoende plantenmateriaal aanwezig. Voor het veredelingswerk wil ik Ir. Ingeborg Westerdijk-Hoks hartelijk bedanken. Voor het opkweken, het verzorgen, en het regelmatig oogsten en verzenden van het plantenmateriaal ben ik Frank Nagelhout zeer erkentelijk.

In het kader van hun bijvakonderzoek hebben Perry Kuiper en Wil Speerstra hun steentje aan het onderzoek bijgedragen. Als HBO- en MBO-stagiaires hebben Jeanette Alkema en Harm-Jan Visser een groot aantal valeriaanmonsters geanalyseerd.

Zonder de specifieke kennis op hun vakgebied van degenen die als mede-auteur aan de verschillende publicaties hebben meegewerkt, zou dit proefschrift niet in deze vorm tot stand zijn gekomen.

Bei Christa und Gerolf Tittel möchte ich mich herzlich für die vielen Diskussionen über die phytochemische Analyse und ihre Methoden bedanken, Bei unseren regelmäßigen Besuchen in Gräfelfing wurden viele Probleme diskutiert und gelöst bei einem original bayrischen Weißbier oder einem guten Schoppen Frankenwein.

De leden van de promotiecommissie, Prof. Dr. R. Verpoorte, Prof. Dr. K.-H. Kubeczka en Prof. Dr. W. Kubelka, ben ik zeer erkentelijk voor het kritisch beoordelen van het manuscript; Rob Verpoorte vooral voor de goede suggesties waardoor hoofdstuk 2 toch een leesbaar geheel is geworden. Bei Karl-Heinz Kubeczka und Wolfgang Kubelka bedanke ich mich herzlich für die Bereitwilligkeit, das Manuskript zu begutachten.

Verder wil ik iedereen, die hier niet met naam genoemd wordt, maar toch - in welke vorm dan ook - een bijdrage aan dit proefschrift heeft geleverd, daarvoor hartelijk danken.

Tot slot wil ik de leden van de werkgroep Farmaceutische Biologie die door de jaren heen bij de werkgroep werkzaam zijn geweest, of dat nog zijn, bedanken voor de prettige samenwerking.