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Review

# Determination of valepotriates

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**Abstract**

In this paper an overview is given of qualitative and quantitative methods of analysis used for valepotriates. Methods like spectophotometry, titrimetry, TLC, GC, HPLC, MS, CE as well as *p*-SFC have been applied. Today HPLC is the method of choice. The usefulness of the individual methods are discussed.


*Keywords:* Reviews; Valepotriates; Valerian; Valerianaceae

## Contents



## 1. Introduction

Valepotriates (valeriana-epoxy-triesters) are natural products that, from a chemical point of view belong to the iridoids. Iridoids are cyclopentan-c-pyran monoterpenoids that are found in a large number of plant families, often in the form of glycosides [1]. The structure of the 10 carbon basic skeleton of the iridoids was established in 1958 [2]. Valepotriates are common in the plant family Valerianaceae [3].

In the 1960s, Thies and co-workers [4,5] isolated

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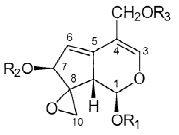
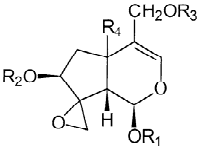


| | $R_1$ | $R_2$ | $R_3$ | |
|---|---|---|---|---|
| **1** | Iv | Iv | Ac | Valtrate |
| **2** | Iv | Ac | Iv | Isovaltrate |
| **3** | Aiv | Iv | Ac | Acevaltrate |
| **4** | Iv | Ac | Ac | Diavaltrate |
| **5** | Iv | Aiv | Ac | Homoacevaltrate |
| **6** | Miv | Iv | Ac | 1-Homovaltrate |
| **7** | Iv | Ac | Miv | 7-Homovaltrate |
| **8** | Iv | Ac | Aiv | 11-Acevaltrate |
| **9** | Iv | Hiv | Ac | Hydroxyvaltrate |
| **10** | Cr | Iv | Ac | 1-Seneciovaltrate (Isohomoacevaltrate) |
| **11** | Iv | H | Iv | Deacetylisovaltrate |

Fig. 1. Valepotriates (diene type).

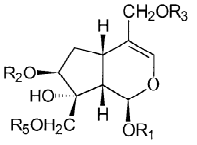
the first valepotriates, as representatives of an, at that time, novel group of natural products from subterranean parts of *V. wallichii*. Based on their chemical structure, valepotriates can be divided into four main groups, namely the diene type (Fig. 1), the monoene type (Fig. 2), the valtrate-hydrine type (Fig. 3), and the desoxy monoene type of valepotriates (Fig. 4) [6]. In addition there exist a number of valepotriates that do not belong to these four types (Fig. 5). In all of these groups variation in side chain substituents are found (Fig. 6).

Valepotriates are instable compounds: they are thermolabile and decompose rapidly under acidic or alkaline conditions in water, as well as in alcoholic solutions. In anhydrous methanol, however, and stored at 20 °C, the diene valepotriates seem to be relatively stable. Dissolved in methanol or ethanol, with only a small amount of water and stored at



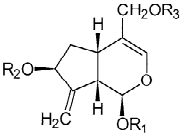
| | $R_1$ | $R_2$ | $R_3$ | $R_4$ | |
|---|---|---|---|---|---|
| **12** | Iv | Ac | Iv | H | Didrovaltrate |
| **13** | Iv | Iv | Ac | H | Isodidrovaltrate |
| **14** | Iv | Ac | Iiv | OH | IVHD valtrate |
| **15** | Miv | Ac | Iv | H | Homodidrovaltrate |
| **16** | Ac | Iv | Aiv | H | AHD valtrate |

Fig. 2. Valepotriates (monoene type).



| | $R_1$ | $R_2$ | $R_3$ | $R_5$ | |
|---|---|---|---|---|---|
| **17a** | Iv | Iv | Iv | Ac | Valtrate hydrine B1 |
| **17b** | Iv | Iv | Ac | Ac | Valtrate hydrine B2 |
| **17c** | Biv | Iv | Iv | Ac | Valtrate hydrine B3 |
| **17d** | Iv | Iv | Ac | Iv | Valtrate hydrine B4 |
| **17e** | Iv | Iv | Miv | Ac | Valtrate hydrine B5a |
| **17f** | Iv | Miv | Iv | Ac | Valtrate hydrine B5b |
| **17g** | Iv | Miv | Ac | Ac | Valtrate hydrine B6a |
| **17h** | Miv | Iv | Ac | Ac | Valtrate hydrine B6b |
| **17i** | Cr | Iv | Iv | Ac | Valtrate hydrine B7 |
| **17j** | Aiv | Iv | Iv | Ac | Valtrate hydrine B8 |

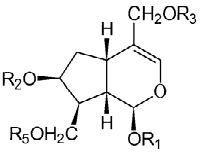
Fig. 3. Valepotriate hydrines.



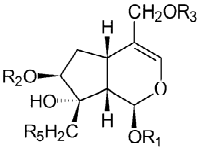
| | $R_1$ | $R_2$ | $R_3$ | |
|---|---|---|---|---|
| **18** | Iv | Ac | Iv | 8,11-Desoxididrovaltrate |
| **19** | Miv | Ac | Iv | 8,11-Desoxihomodidrovaltrate |

Fig. 4. Desoxy monoene valepotriates.

room temperature, gives 90% decomposition within a few weeks [7]. The main decomposition products of the valepotriates are the yellow-coloured baldrinals. Losing the substituted groups on $C_1$ and $C_7$ of valtrate and acevaltrate, and forming of an aldehyde



| | $R_1$ | $R_2$ | $R_3$ | $R_5$ | |
|---|---|---|---|---|---|
| **20** | Iv | H | Glu | H | Patrinoside |



| | $R_1$ | $R_2$ | $R_3$ | $R_5$ | |
|---|---|---|---|---|---|
| **21** | Iv | Iv | Ac | Cl | Valechlorine |
| **22** | Iv | H | Glu | H | Valerosidate |

Fig. 5. Other valepotriates.

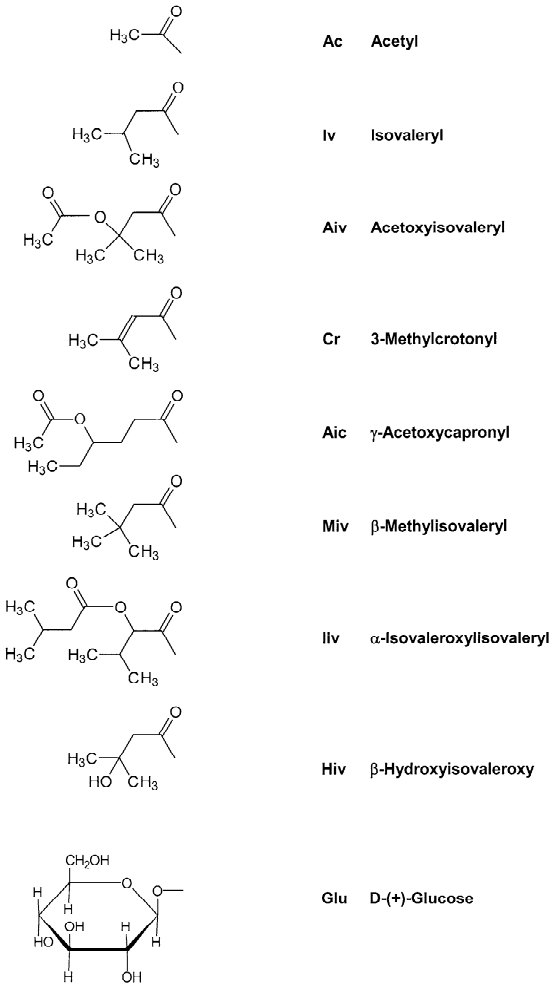
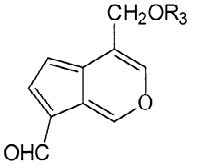



Fig. 6. Side chain substitutions found in valepotriates as present in *Valeriana* species, $R_1$–$R_3$ can be:.

group on $C_{10}$ yields baldrinal; homobaldrinal originates by losing an acetyl and an isovaleryl group on $C_1$ and $C_7$ from isovaltrate (Figs. 1 and 7) [8,9]. The baldrinals (Fig. 7) are chemically reactive and may subsequently form polymers [7,8].

The family Valerianaceae is composed of 13–17 genera (different authors give different numbers), and contains 300–400 species [3,10]. In Table 1, an overview is given of genera in the family Valerianaceae and a number of species of the genus *Valeriana*, which contain valepotriates. High amounts are present in *V. edulis* (8.0–12.0%) and *V. wallichii* (1.8–3.5%), both species are being used as




| | $R_3$ | |
|---|---|---|
| **23** | Ac | Baldrinal |
| **24** | Iv | Homobaldrinal |
| **25** | H | Deacylbaldrinal |

Fig. 7. Baldrinals (artifacts).

starting material for the preparation of valepotriate standarised phytomedicines.

Plant cell culture may offer the alternative for a controlled production of natural products. Often low quantities of the desired compounds are accumulated by undifferentiated cell cultures. The initiation of organ cultures stands a better chance in obtaining higher amounts of natural products [11]. In this context a good example is the accumulation of valepotriates up to 0.83% in root cultures of *V. wallichii* (Table 1) [12].

The genus *Valeriana* is most extensively investigated and contains about 230 species. The majority of representatives of this genus are 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. and Gray ssp. *procera* (H.B.K.) F.G. Meyer (synonym *V. mexicana* DC; Mexican valerian) [30,36]. *V. officinalis* L *s.l.* (valerian) is included in the European Pharmacopoeia (2002, 4th ed.). In Europe this species is cultivated on a large scale for the preparation of phytomedicines, prepared from the subterranean parts [7]. *V. wallichii* DC, the Indian or Pakistani valerian, is native to the Himalayas [8]. *V. edulis* Nutt. ex Torr. and Gray ssp. *procera* (H.B.K.) F.G. Meyer originates from Central America. Other well-known and studied genera are *Centranthus* DC and *Nardostachys* DC (Table 1) [3,13,38–40].

The Greek and the Roman physicians already used valerian root as a diuretic, pain relieving and spasmolytic agent. Nowadays, valerian preparations are used primarily as a mild sedative, to treat light forms of neurasthenia and emotional stress [41,42].

Table 1
Valepotriates in different Valerianaceae genera[a]

| Valepotriates (DW, %) | Roots and rhizomes | Leaves | Stems | Flowers | Biotech.[b] | References |
|---|---|---|---|---|---|---|
| *Valeriana* | + | | | | | [3,6,13–25,76] |
| Several species | | | | | | |
| *V. officinalis* | 0.3–1.7 | | | | + | [24,26–29,88] |
| *V. wallichil* | 1.8–3.5 | | | | 0.83 | [12,30] |
| *V. edulis* | 8.0–12.0 | | | | + | [6] |
| *V. kilimandschari* | 5.2 | 5.9 | 3.2 | 3.8 | | [31] |
| *V. alliarilfolia* | + | + | + | | + | [3,14,33] |
| *V. dioicia* | 0.2 | + | + | | | [33,88] |
| *V. tripteris* | 0.3 | + | + | | | [33,88] |
| *Centranthus* | + | + | + | | + | [6,14,20,24,32–35,39] |
| *Fedia* | + | + | + | | + | [3,14,20,29,32,33] |
| *Phyllactris* | + | | | | | [13,20] |
| *Valerianella* | + | | | | + | [14,15,20,24,29,33] |
| *Belonanthus* | + | | | | | [6,13] |
| *Patrinia* | – | | | | | [3,33] |
| *Plectritis* | + | | | | | [20] |
| *Nardostachys* | – | | | | | [3,33] |
| *Stangea* | + | | | | | [36] |

+ valepotriates present, exact value unknown.
[a] For a detailed overview see Refs. [37,88].
[b] Cell suspension, tissue, callus, and hairy root cultures.

In the past decades numerous studies have been directed to the pharmacological effects of valerian extracts and their isolated constituents [9]. It is however still not clear which constituents are responsible for the sedative action. Characteristic sesquiterpenes, valerenic acid and derivatives, are considered as important. These compounds only occur in *V. officinalis* (Fig. 8B). In addition, the essential oil may contribute to the effect. The role of valepotriates is somewhat controversial. They have been considered as important for biological action and several studies point to pharmacological effects of these compounds. However, due to their low stability, they are often not present in pharmaceutical dosage forms of valerian. They decompose upon storage as well as during the manufacturing process [7]. On the other hand, valepotriates have been found to be cytotoxic, mutagenic and carcinogenic in vitro. Based on their potential toxicity, they may be regarded as unwanted in phytomedicines [43]. The relevance of the probable unsafeness of valepotriates in humans is unclear at present. When included in a taken preparation. they will decompose rapidly in the gastrointestinal tract probably before they are absorbed [9].

Summarising, the availability of reliable analytical procedures for valepotriates is necessary for quality control and identification of the crude drug (each *Valeriana* species has its own characteristic valepotriate profile, see Fig. 8A–C), for the control of manufacturing processes and for quality control of phytomedicines prepared from valerian. In the latter case a suitable analysis can be used to prove the absence of the potentially cytotoxic valepotriates.

In this paper an overview is given of qualitative and quantitative methods of analysis used for valepotriates and the currently available state-of-the-art methods are presented. We start with the description of a general method of sample preparation and procedures for the isolation of valepotriates to obtain reference compounds.

## 2. Sample preparation for valepotriate analysis

A general method for the extraction of plant material is described below. The method is included in the European Pharmacopoeia (2002, 4th ed.). For phytomedicines an extraction method was developed by the authors [7]. Both extraction methods allow high-performance liquid chromatography (HPLC)

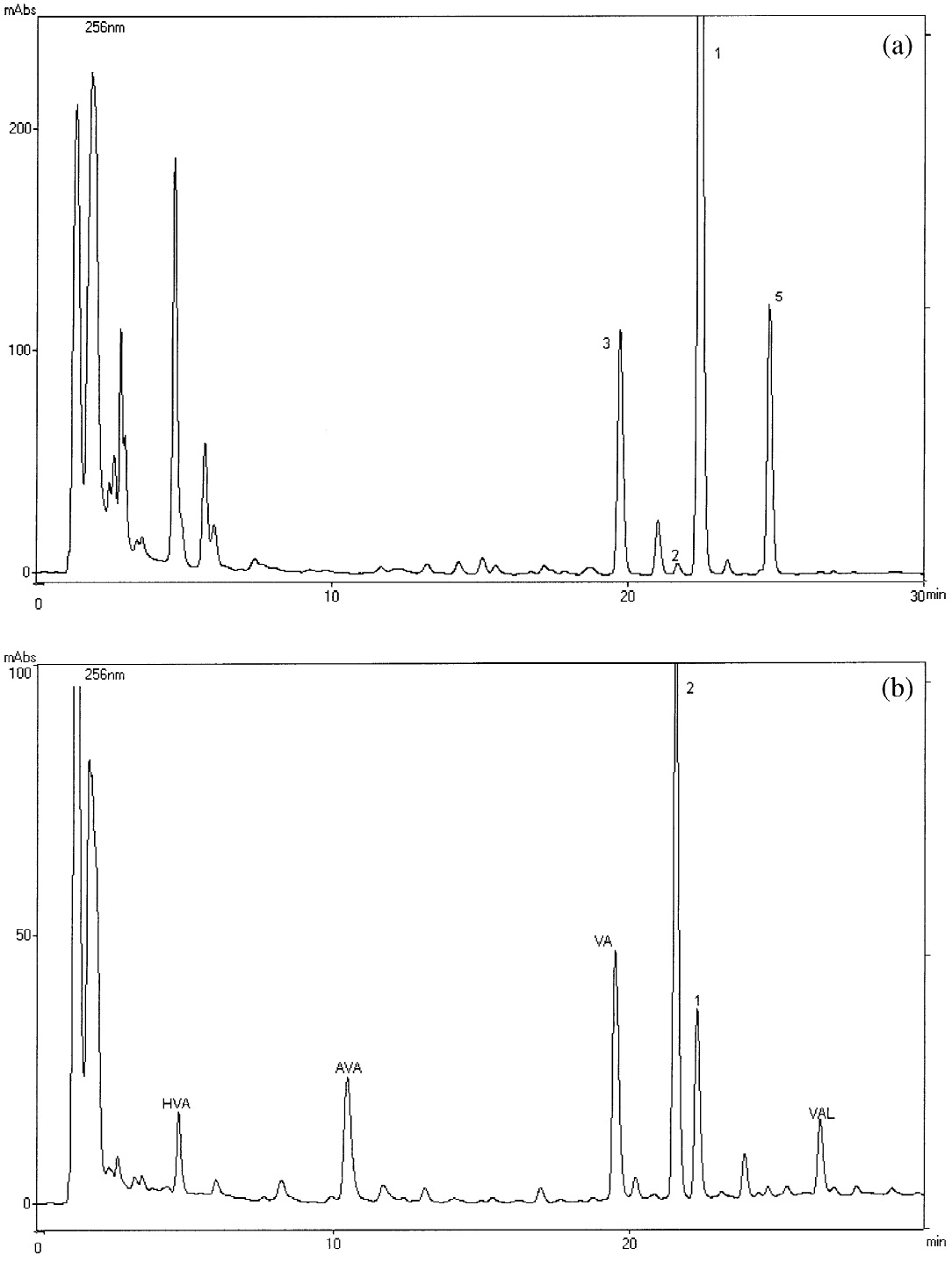



Fig. 8. HPLC chromatograms of *Centranthus ruber* (A); present are acevaltrate (3), isovaltrate (2), valtrate (1), and homoacevaltrate (5); of *Valeriana officinalis* (B); present are acevaltrate (3), isovaltrate (2), and valtrate (1), also present are hydroxyvalerenic acid (HVA), acetoxyvalerenic acid (AVA), valerenic acid (VA), and valerenal (VAL); and of *Valeriana alliaiifolia*, present are the valtrate hydrines B3 (17c), B2 (17b), B6a and B6b (17 g, h), acevaltrate (3) valtrate hydrine B8 (17j), isovaltrate (2), valtrate (1), valtrate hydrine B7 (17i), B4 (17d), B1 (17a) and homoacevaltrate (5).

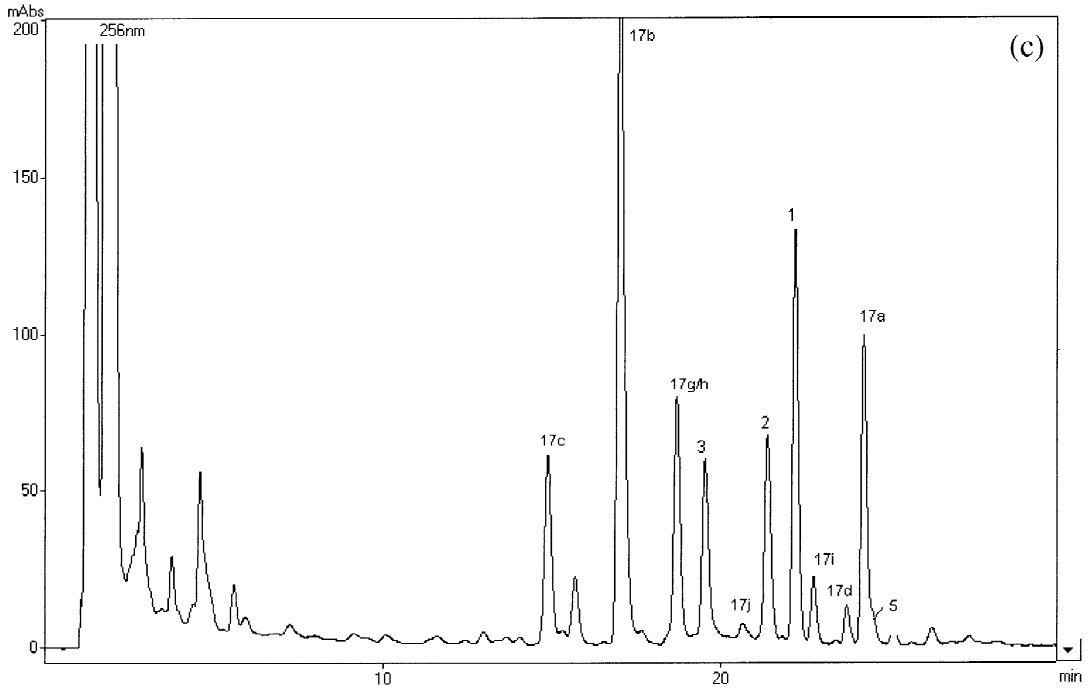



Fig. 8. (*continued*)

analysis of valepotriates as well as valerenic acid derivatives.

### *2.1. Plant material*

Extract 1.5 g ground dried plant material (1 mm) with 20 ml anhydrous methanol and heat on a water-bath under a reflux condenser for 30 min. Allow to cool and filter into a 50 ml volumetric flask. Add another 20 ml of anhydrous methanol and heat on a water-bath under the reflux condenser for 15 min. Allow to cool and filter in the same volumetric flask. Dilute to 50 ml with anhydrous methanol [44].

### *2.2. Phytomedicines*

Tinctures can be analysed as such. For capsules the procedure is as follows: cut one capsule and extract the material with three portions of 30 ml anhydrous methanol for 5 min in an ultrasonic bath. The extracts are filtered into a volumetric flask and the volume is adjusted to 100 ml with anhydrous methanol. For tablets: grind five coated tablets, and extract twice with 10 ml anhydrous methanol for 5 min in an ultrasonic bath. The extracts are filtered into a volumetric flask and the volume is adjusted to 25 ml with anhydrous methanol.

All samples must be filtered through a 45-μm HPLC filter (DynaGard HPLC filter, Microgon, Laguna Hills, CA, USA) before analysis [7].

## 3. Isolation procedures for valepotriates and structure elucidation

Several different isolation procedures for valepotriates to obtain reference compounds have been published. Commonly, extracts of the plant material are prepared with dichloromethane and analysed by straight-phase HPLC. For the isolation of valepotriates preparative thin-layer chromatography (TLC) (also for the artifacts baldrinal and homobaldrinal) [14,45–48], and semipreparative HPLC and column chromatography (CC) have been used [15].

For identification purposes and for structure elucidation standard spectroscopic methods (infrared [IR],

Table 2
Spectral data of the most frequently occuring valepotriates as found in the literature

| Valepotriate type | Formula | MW | UV-max (nm, lit.) | Melting point (°C) | IR | NMR | MS | References |
|---|---|---|---|---|---|---|---|---|
| *Diene type* | | | | | | | | |
| 1. Valtrate | $C_{22}H_{30}O_8$ | 422 | 256 (MeOH) | oil | IR | NMR | MS | [1,3,5,15,47,49–52,76] |
| 2. Isovaltrate | $C_{22}H_{30}O_8$ | 422 | 256 (MeOH) | oil | IR | NMR | MS | [1,3,5,15,49,50,53] |
| 3. Acevaltrate | $C_{24}H_{32}O_{10}$ | 480 | | 83–84 | IR | NMR | MS | [1,3,14,47,51,52,54,55,76] |
| 4. Diavaltrate | $C_{19}H_{24}O_{10}$ | 380 | 256 | 70–71 | IR | NMR | MS | [3,15,50,52,54,55,57] |
| 5. Homoacevaltrate | $C_{24}H_{32}O_{10}$ | 480 | | 82–83 | | NMR | | [1,50,52] |
| 6. 1-Homovaltrate | $C_{23}H_{36}O_8$ | 436 | | | IR | NMR | | [3,54] |
| 7. 7-Homovaltrate | $C_{23}H_{32}O_8$ | 436 | | | IR | NMR | MS | [3,54,55] |
| 8. 11-acevaltrate | $C_{24}H_{32}O_{10}$ | 480 | | | | | | |
| 9. Hydroxyvaltrate | $C_{22}H_{30}O_9$ | 438 | | | | NMR | | |
| 10. 1-Seneciovaltrate | $C_{22}H_{28}O_8$ | 420 | | | IR | NMR | | [54,56] |
| 11. Desacetylisovaltrate | $C_{20}H_{28}O_7$ | 380 | | | IR | NMR | | [54] |
| *Monoene type* | | | | | | | | |
| 12. Didrovaltrate | $C_{22}H_{32}O_8$ | 424 | 210 | 64–65 | IR | NMR | MS | [1,3,5,15,47,49–51,53,58,76] |
| 13. Isodidrovaltrate | $C_{22}H_{32}O_8$ | 424 | | | IR | NMR | | [1,17,52,58] |
| 14. IVHD-valtrate | $C_{27}H_{40}O_9$ | 540 | 206 | 64–65 | IR | NMR | MS | [1,3,15,50,52–55] |
| 15. Homodidrovatrate | $C_{23}H_{34}O_8$ | 438 | 206 | 50–51 | IR | NMR | MS | [1,15,47,50,51] |
| 16. AHD-valtrate | $C_{24}H_{34}O_{10}$ | 482 | | | IR | NMR | MS | [1,45,52,54,55,76] |
| *Valtrate hydrine type* | | | | | | | | |
| 17. Valtrate hydrine B1–B8 | | | 202/254 | | IR | NMR | MS | [1,15,17,39,49,50,56] |
| *Desoxy monoene type* | | | | | | | | |
| 18. 8,11-desoxididrovoltrate | $C_{22}H_{33}O_8$ | 424 | | 68–70 | IR | NMR | | [1,47,51] |
| 19. 8,11-desoxi-homodidrovaltrate | $C_{23}H_{34}O_7$ | 422 | | | | | | |
| *Other valepotriates* | | | | | | | | |
| 20. Patrinoside | $C_{21}H_{34}O_{11}$ | 462 | | 97–98 | IR | NMR | | [1,39,59] |
| Patrinoside aglycon | $C_{15}H_{24}O_6$ | 300 | | 111–113 | IR | NMR | MS | [1] |
| 21. Valechlorine | $C_{22}H_{31}O_8Cl$ | 458 | 200/254 | 79–80 | IR | NMR | MS | [1,60,61] |
| 22. Valerodisate | $C_{21}H_{34}O_{11}$ | 462 | | 78–80(152) | IR | NMR | MS | [1,23,49,62,63] |
| *Baldrinals* | | | | | | | | |
| 23. Baldrinal | $C_{12}H_{10}O_4$ | 218 | | 112–113 | IR | NMR | MS | [41,47,49,51,52] |
| 24. Homobaldrinal | $C_{15}H_{16}O_4$ | 260 | | 36–37 | IR | NMR | | [49,64] |
| 25. Deacylbaldrinal | $C_{10}H_8O_3$ | 176 | | | | | | [65] |

nuclear magnetic resonance [NMR], mass spectrometry [MS]) have been used. In Table 2 spectral data of the most frequently occurring valepotriates are given.

## 4. Qualitative analysis

For the qualitative analysis of valepotriates mainly TLC (and sometimes HPLC) has been used. A series of TLC methods for the determination of valepotriates, qualitatively as well as (semi)quantitatively, have been described in the literature. A detailed overview is given in Table 3.

The first complete instruction for quickly testing valerian root for its valepotriates was published in 1969 [69]. 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

Table 3
TLC analysis of valepotriates as found in the literature

| Stationary phase | Mobile phase | Detection | Literature |
|---|---|---|---|
| Silica gel 60 $F_{254}$ [a] | Hexane:2-butane = 8:2 (2-dim.) | Benzidine–HCl | [66] |
| Silica gel $HF_{254}$ | *n*-Hexane:MEK = 4:1 | | [66] |
| Silica gel 60 $F_{254}$ | Benzene:EtOAc = 85:15 | | [66] |
| | Toluene:MEK = 4:1 | Benzidine–HCl | [14] |
| | Toluene:*n*-hexane:MEK = 35:45:20 | | [14] |
| Silica gel G | Petrol ether:$Me_2CO$:EtOAc = 100:8:8 (prep.) | Benzidine–HCl | [45,57] |
| Silica gel G | $CH_2Cl_2$:$Me_2CO$:EtOAc = 50:1:1 (prep.) | | [45,57] |
| Silica gel F60 | *n*-Hexane:MEK = 8:2 | UV (254/366) | [67] |
| Silica gel $HF_{254/366}$ | Benzene:EtOAc = 83:17 | Acetic acid/HCl | [67] |
| | $CH_2Cl_2$:MEK = 98:2 | Hydroxylamine/HCl/NaOH/EtOH | [67] |
| Silica gel $HF_{254}$ | *n*-Hexane:MEK = 8:2 (2×10 cm) | Benzidine–HCl | [13] |
| $Al_2O_3$ (neutral) | *n*-Hexane:MEK = 7:3 (1×13 cm) | Benzidine–HCl | [13] |
| Silica gel 60 $F_{254}$ | *n*-Hexane:MEK = 8:2 | UV, DNPH | [33] |
| | $CH_2Cl_2$:$Me_2CO$:EtOAc = 48:1:1 (prep.) | | [33] |
| Silica gel | Benzene:EtOAc = 9:1 | 0.1% DNPH in HCl (25%):$H_2SO_4$ = 1:1 | [68] |
| | Light petrol:EtOAc = 8:2 | | [68] |
| | Diethyl ether:hexane = 5:5 | | [68] |
| | Light petrol:$Me_2CO$ = 8:2 | | [68] |
| Silica gel $GF_{254}$ | Hexane:MEK = 8:2 (2×10 cm) | UV 254/benzidine–HCl (110 °C) | [69] |
| | | HCl (6 N) | [70] |
| | | HCl (25%): $H_2SO_4$ = 1:1 | [5] |
| | | Antimony trichloride (22% in chloroform) | [5,70] |
| Silica gel GF $F_{254}$ | Benzene:EtOAc = 9:1 | 22% antimony chloride/DNPH (105 °C) | [34] |
| | Light petrol:EtOAc = 8:2 | | [34] |
| | Diethyl ether:Hexane = 5:5 | | [34] |
| | Light petrol:$Me_2CO$ = 8:2 | | [36] |
| Silica gel $GF_{254}$ | Hexane:MEK = 9:1 (2-dim.) | UV 254/DNPH (5 min 105 °C) | [71] |
| | Hexane:MEK = 8:2 (2-dim.) | | [71] |
| | $CH_2Cl_2$:MEK = 9:1 (2-dim.) | | [71] |
| | Toluene:EtOAc:MEK = 80:15:5 (unsat., 13 cm) | UV 254/DNPH (5 min 105 °C) | [53] |
| RP-$C_{18}$ (HPTLC) | MeOH:water = 8:2 | Ammonia gas/UV/279 nm/255 nm/425 nm | [72,73] |
| Silica gel 60 $F_{254}$ | $CH_2Cl_2$:$Me_2CO$:EtOAc = 48:1:1 | | [72] |
| | $CH_2Cl_2$:MEK-98:2 | | [72] |
| Silica gel 60 $F_{254}$ | *n*-Hexane:MEK = 8:2 | UV (254 nm)/DNPH/NBP | [74] |
| Silica gel 60 $F_{254}$ | *n*-Hexane:MEK = 8:2 | NBP (3% in acetone, 40 °C 90 min) | [74] |
| Silica gel 60 $F_{254}$ | Benzene:EtOAC = −83:17 | UV (254/366)/DNPH | [48] |
| Silica gel 60 $F_{254}$ | Toluene:EtOAc:MEK = 80:15:5 | DNP/NBP | [75] |
| Silica gel HG 60 $F_{254}$ | Toluene:EtOAc = 78:22 | UV (254 nm)/acetic acid/HCl | [56] |
| Silica gel $GF_{254}$ | $CHCl_3$:MeOH 50:0.5 | UV (254 nm) | [76] |
| | $CHCl_3$:MeOH 8:2 | | [76] |

DNPH, 2,4-dinitrophenylhydrazine; MEK, methyl ethyl ketone; NBP, 4-(4-nitrobenzyl)-pyridine.
[a] Impregnated with 1% polyethylene in MeOH.

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 visualised 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 [69]. 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 as an improvement if compared with older reagents. Spraying with 6 *M* hydrochloric acid yielded an instable blue colour with valtrate and acevaltrate [70]. Also a mixture of equal volumes of hydrochloric acid (25%) and glacial acetic acid coloured the diene valepotriates only [5]. Antimony trichloride (22%, w/v) in chloroform did not give clear colours in daylight [5,70].

Benzidine is a carcinogenic agent; as a substitute, a solution of 0.1% 2,4-dinitrophenylhydrazine in hydrochloric acid (25%)–glacial acetic acid 1:1 as a spraying reagent was developed [68]. The disadvantage was a yellow background was obtained and the fluorescence was less; advantageously, 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 [68].

Laufer and co-workers [34] developed an improved TLC analysis for valepotriates, based on the method of Stahl and Schild [68], using the same stationary phase and the same mobile phase. The reference solution was also the same, 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 isovaleroxyhydroxydidrovaltrate (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).

A two-dimensional TLC for the separation of valepotriates was applied, 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 [71].

Later, a better separation of the valepotriates was obtained, 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 [53].

Another TLC method allows determination of valepotriates 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 [69]. 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 the 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 10-fold more sensitive than the detection with anisaldehyde reagent [73].

The reaction with 4-(4-nitrobenzyl)pyridine (NBP) has been used for the quantitative determination of valepotriates [74]. 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.

A method yielding a good separation and spot detection was developed in our laboratory by Hazelhoff et al., using pre-coated TLC plates (silica gel $F_{254}$, 20×20 cm, Merck Darmstadt, Germany)

Table 4
TLC data of the most common valepotriates and baldrinals on silica gel 60 $F_{254}$ using the method of Hazelhoff et al. [75]

| Component | $R_F$ (15 cm) | Daylight | 254 nm | DNPH | NBP |
|---|---|---|---|---|---|
| IVHD | 0.31 | n.d. | – | Light-blue | Grey |
| Acevaltrate | 0.49 | n.d. | + | Blue | Grey–yellow |
| Homobaldrinal | 0.50 | Yellow | + | Grey–brown | Yellow |
| Baldrinal | 0.23 | Yellow | + | Brown | Yellow |
| Didrovaltrate | 0.53 | n.d. | – | Brown | n.d. |
| Valtrate/isovaltrate | 0.63 | n.d. | + | Dark-blue | Grey–yellow |

Mobile phase: Toluene:EtOAc:MEK = 80:15:5; DNPH = 2,4-dinitrophenylhydrazine; NBP = 4-(4-nitrobenzyl)-pyridine; n.d. = not detected.

and toluene–ethyl acetate–methyl ethyl ketone = 80:15:5 as the mobile phase were applied (Table 4) [75]. The detection solutions used are:

- 2,4-Dinitrophenylhydrazine (DNPH); 0.2 g DNPH is dissolved in 20 ml MeOH, then a mixture of 40 ml acetic acid and 40 ml of a 25% solution of hydrochloric acid is added. The plate was heated for 10 min at 110 °C.
- 4-(4-nitrobenzyl)-pyridine (NBP); 3 g NBP is dissolved in 100 ml acetone. The plate was heated for 10 min at 110 °C. A detailed overview of the results is given in Table 4.

Nowadays TLC is still used for a rapid qualitative valepotriate analysis of large numbers of samples. For recent reviews see Refs. [3,37].

## 5. Quantitative analysis

For quantitative analysis of valepotriates spectrophotometric and titrimetric methods, TLC, gas chromatography (GC), HPLC as well as HPLC/MS have been applied.

### 5.1. Spectrophotometry

The first procedure for a quantitative determination of valepotriates has been described in 1968. The 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 was described [64]. 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 [64]. The identity reaction for *V. officinalis*, as described in the European Pharmacopoeia 2002, 4th ed., is derived from this earlier work [44].

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. Later a spectrophotometric method by which all valepotriates can be determined is described [77]. 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 [74]. Of a sample and a reference solution (30 mg valtrate or didrovaltrate in 10 ml methanol), 50 μl were incubated in a water-bath at 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 *M*; 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.

### 5.2. Titrimetry

The first titrimetric determination of valepotriates has also been described in 1968 [64], 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 [78] published an improved method with respect to time. The titration of valepotriates was by opening the epoxide ring with hydrobromic acid; the reaction mixture was rapidly titrated with 0.01 *M* sodium acetate solution. Just before the end of the titration 5 ml of a Crystal Violet solution was added as an indicator. The titration was continued until a blue colour appeared.

### *5.3. Thin layer chromatography (TLC)*

Quantitative TLC methods for the analysis of valepotriates have been published in 1979 and 1981 [53,72]. Acevaltrate and (iso)-valtrate were detected under the influence of UV light. The measurements were carried out using a Zeiss chromatogram spectrophotometer (PMQ II) at 254 nm [53]. In the second publication the use of HPTLC–RP-18 and HPTLC–silica gel 60 plates was described. Detection of a number of valepotriates and baldrinals was carried out with a TLC/HPTLC scanner. Also the retention times with three different eluents are given [72]. Quantitative TLC is replaced by HPLC nowadays.

### *5.4. Gas chromatography (GC)*

In the 1970s two GC methods for the analysis of valepotriates were published [71,79]. The first method was for the determination of valtrate, acevaltrate and didrovaltrate as well as baldrinal. Two different stationary phases, namely 2% OV-1 and 3% OV-17 were used [71].

In a second method, 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 *M* methanolic potassium hydroxide solution. 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 [81]. These GC methods are laborious, no longer used nowadays and are replaced by HPLC methods.

### *5.5. High-performance liquid chromatography (HPLC)*

A number quantitative HPLC methods for analysis of valepotriates using straight-phase (SI) and reversed-phase (RP-8 and RP-18) have been published (Table 5). HPLC methods are also applied to monitor a total profile of valepotriates as present in samples. HPLC analysis is used for both crude plant material and phytomedicines (tinctures, capsules and coated tablets).

The first HPLC methods for the determination of valepotriates were described in 1978 [80,81]. 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×4 mm I.D. or LiChrosorb Si 100, 35 cm×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 [77], 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 was presented in 1979 [53]. A Spherisorb Silica S5W column, 25 cm×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 [83] 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×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×3 7 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.

Table 5
HPLC analysis of valepotriates and baldrinals as found in the literature

| Stationary phase | Mobile phase | Detection | Literature |
|---|---|---|---|
| *Straight-phase* | | | |
| Nucleosil 50 (250×4 mm I.D.) | *n*-Hexane:ethyl acetate=20:3 | IR and UV | [48,80] |
| Lichrosorb Si 100 (5 μm) | $CH_2Cl_2$:EtOAc=20:1 | UV (254) | [81] |
| Spherisorb silica S5W (250×4.6 mm, 5 μm) | Hexane:methanol=92:8 | UV (254 nm) | [53,75,82] |
| Partisil silica (250×3 mm. 5 μm) | *n*-Hexane:ethyl acetate=90:10 | | [83] |
| | *n*-Hexane:ethyl acetate=95:5 | | [83] |
| | *n*-Hexane:ethanol=99.5:0.5 | | [83] |
| *Reversed-phase* | | | |
| Spherisorb ODS (250×3 mm, 5 μm) | Methanol:water=50:50 | | [83] |
| | Acetonitrile:water=60:40 | UV (206 and 256 nm) | [83] |
| Bondapack $C_{18}$ (300×3.9 mm) | A=methanol:water=2:8; B=methanol:water=8:2 | UV (254 nm) | [31] |
| | Methanol:water=8:2 | UV (208 nm) | [31] |
| Ultrasphere ODS (250×10 mm, 5 μm) | Acetonitrile:water=7:3 | UV (206 nm) | [84] |
| | A=methanol:water=6:4; B=methanol:water=9:1 | UV (254) | [84] |
| Lichrospher 100 CH (250×4 mm, 5 μm) | A=methanol:water=4:6; B=methanol:water=9:1 | UV (208 and 254 nm) | [85] |
| Lichrosorb RP-18 | Methanol:water 8:2 (0.5% phosphoric acid) | UV (255 nm) | [86] |
| Nucleosil $C_{18}$ (250×4 mm, 5 μm) | A=methanol:water=9:1; B=methanol:water=4:6 | UV (206 and 254 nm) | [15] |
| Nucleosil $C_{18}$ (250×4 mm, 5 μm) | Methanol:water 7:3 | UV (208 nm) | [26] |
| Nucleosil $C_{18}$ (250×4 mm, 5 μm) | A=methanol:water=9:1; B=methanol:water=6:4 | UV (208 and 254 nm) | [26] |
| Zorbax RP-18 ODS (250×4.6 mm) | Acetonitrile:water=62:38 | UV (254 nm) | [55] |
| Lichrosorb RP-18 (250×4 mm, 10 μm) | Methanol:water=3:7 | UV (220 nm) | [87] |
| Nova-Pak C-18 (150×3.9 mm, 4 μm) | Acetonitrile:water=55:45 | UV (254 nm) | [28] |
| ApexPrep ODS-2 (250×4.6, 8 μm) | Methanol:$H_2O$ (0.5% $H_3PO_4$, pH 2)=8:2 | UV (254 nm) | [15,88] |
| *Prep. HPLC* | | | |
| Lichrosorb Si 100 (350×8 mm) | *n*-Hexane:ethyl acetate=20:3 | UV | [48] |
| Nucleosil 100-7 RP-18 (250×21 mm) | Acetonitrile:water=55:45 | UV | [15] |
| Altex Ultrasphere ODS (250×10 mm) | Acetonitrile:water 7:3 | UV 206 nm | [89] |
| Altex Ultrasphere ODS (250×10 mm) | A=methanol:water=6:4; B=methanol:water=9:1 | UV 254 nm | [89] |

Reversed-phase HPLC with gradient elution was used for the determination of diene valepotriates [31]. A Waters Bondapak $C_{18}$ column, 30 cm×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 for 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 and co-workers [89]. The authors used an Altex Ultrasphere ODS preparative column, 25 cm×10 mm, particle size 5 μm, equipped with an Altex guard column (45×4.6 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 for 15 min. Detection of the dienes was at 254 nm.

Chavadej and co-workers 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 [85].

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 [86].

Gränicher and co-workers applied the system of Förster [89], but with some modifications. They used a Nucleosil $C_{18}$ column (25 cm×4 mm I.D., 5 μm) fitted with a Nucleosil $C_{18}$ guard column (30×4 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 isocratically with 60% A for 15 min, and a linear gradient to 90% A for 22 min. The monoene valepotriates were eluted with 60% A for 27 min, followed by a linear gradient to 80% A for 14 min [26].

A state-of-the-art HPLC analysis for valepotriates is the separation on a $C_{18}$ RP column followed by diode array detector (DAD). This method was developed by the authors and is now included in the European Pharmacopoeia (2002, 4th ed.). The standard procedure is as follows: Isco HPLC pump 2350, an Isco low pressure gradient mixer 2360 (Isco, Lincoln, NE, USA), a Kontron autosampler 360 (Kontron Instruments, Milan, Italy) and a Shimadzu SPDM6A-diode array detector (Shimadzu Europe, Duisburg, Germany). The chromatographic conditions are: 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 contain 1 m*M* phosphoric acid); elution programme, first isocratic at 55% A and 45% B for 5 min, then a linear gradient to 100% B for 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 wavelength, 200–600 nm; band width, 2 nm; spectrum absolute scale (mAbs), 10–500; normalisation threshold, 10 mAbs; analyzing temperature, 25 °C [7].

HPLC/MS analysis has been performed with a microHPLC system with a flow-rate of 250 μl $min^{-1}$ to obtain a flow-rate of ca. 4 μl $min^{-1}$ through the analytical column. A pre-injection split was generated by diverting most of the solvent, via a three-way union to a 3 μm Nucleosil $C_{18}$ column (120 mm × 2.1 mm I.D). The other outlet of the union was linked to a Rheodyne 7520 injection valve, with a 500 nl loop, which was coupled directly to a Fusica™-C column (300 mm × 0.32 mm I.D.) packed with a 5 μm $C_{18}$ support. The mobile phase consisted of 70% methanol in 1% aqueous acetic acid in the presence of a 1% glycerol matrix. The column was connected via fused-silica tubing (0.5 m × 50 μm I.D.) and a Jeol frit fast atom bombardment (FAB) to the HPLC/MS interface [3].

Fig. 9 shows a characteristic HPLC profile of a mixture of valepotriates, i.e. three diene valepotriates, two monoene valepotriates, and two baldrinals. As can be seen, all compounds are well separated. In Table 6, the reference valerian compounds, separated with the HPLC system, are listed with their respective retention times, capacity factors and UV maxima. In Fig. 10 the UV spectra of the reference compounds are given; a total of three different UV maxima were found: 256 nm for valtrate, isovaltrate and acevaltrate; 203 nm for didrovaltrate and isovaleroxyhydroxydidrovaltrate (IVHD) and, 230 and 246 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. This is an improvement as compared with previously published analytical procedures, that all require more than one run.

## 6. Final remarks

Two recently developed methods of analysis are capillary electrophoresis (CE) and packed-colum supercritical liquid chromatography (*p*-SFC).

Good separation of valepotriates using capillary electrophoresis (CE) is not possible at the moment. The valepotriates are lipophilic substances and for CE separation the molecules should be either polar or easily be derivatised to a polar compound [90]. Also the use of a modifier might make it possible to develop a suitable method of valepotriate separation by CE.

Packed-column supercritical fluid chromatography (*p*-SFC) of low to medium polarity natural products of plant origin, e.g., valepotriates, using UV-spectroscopy as the detection mode was recently reported by Bicchi and co-workers [91]. They compared the above discussed HPLC method [7] with a *p*-SFC analysis. One of the important results is that *p*-SFC reduced the time of analysis to less than one fourth for the valepotriates valtrate and isovaltrate. The

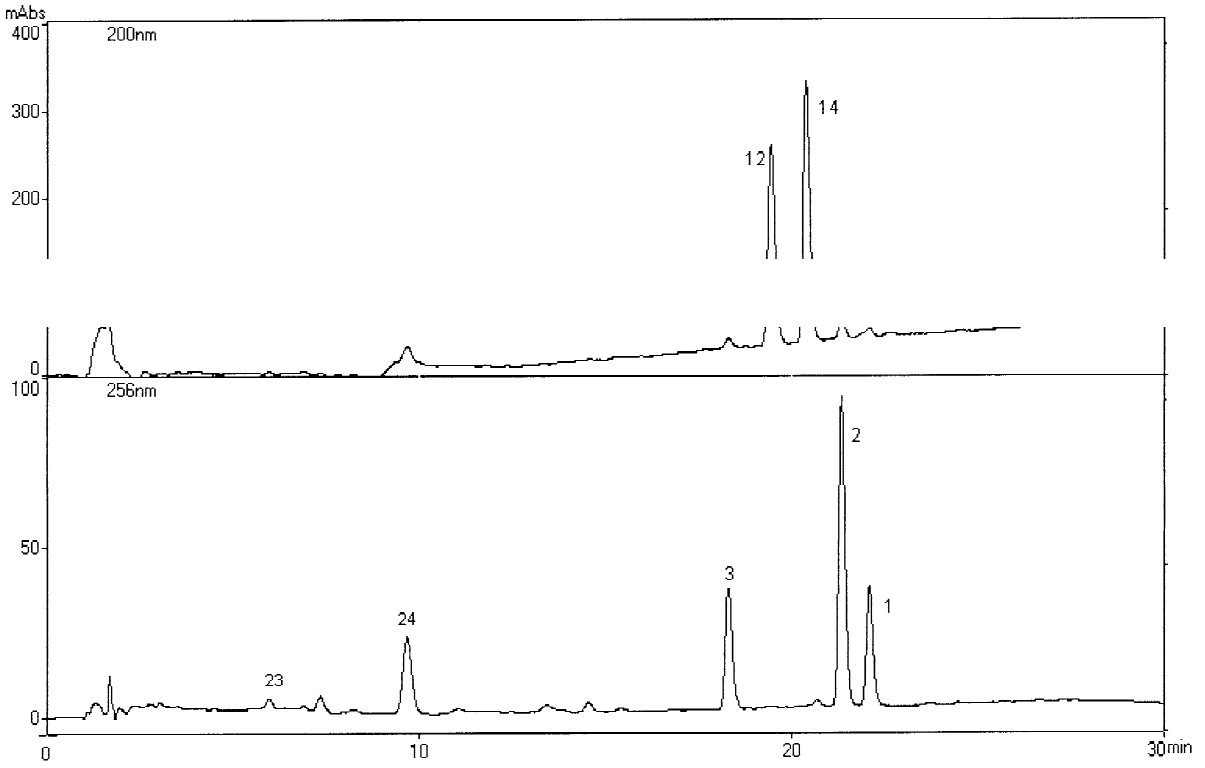



Fig. 9. HPLC/UV–Vis chromatogram at 200 and 256 nm. Present are didrovaltrate (13), isovaleroxyhydroxydidrovaltrate (IVHD-valtrate, 14), baldrinal (23), homobaldrinal (24), acevaltrate (3), isovaltrate (2), and valtrate (1).

results with both techniques are comparable although *p*-SFC % values and standard deviations are slightly higher then those obtained by our HPLC method.

It can be concluded that for the qualitative analysis of valepotriate containing phytomedicines and *Valeriana* plant material, TLC is a suitable method. Large numbers of samples can be rapidly analysed. When more accurate qualitative analysis is required,

Table 6
Reference valerian compounds separated using the on-line HPLC system, with their respective retention time, capacity factor and UV-maximum [7]

| Compound | Retention time (min) | Capacity factor ($k'$)[a] | UV maximum (nm) |
|---|---|---|---|
| Baldrinal | 4.43 | 2.20 | 230, 246, 291, 424 |
| Homobaldrinal | 9.76 | 6.05 | 230, 246, 291, 424 |
| Acevaltrate | 18.32 | 12.23 | 256, 259 |
| Didrovaltrate | 19.47 | 13.06 | 203 |
| IVHD | 20.43 | 13.75 | 203 |
| Isovaltrate | 21.38 | 14.44 | 256, 259 |
| Valtrate | 22.11 | 14.96 | 256, 259 |

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

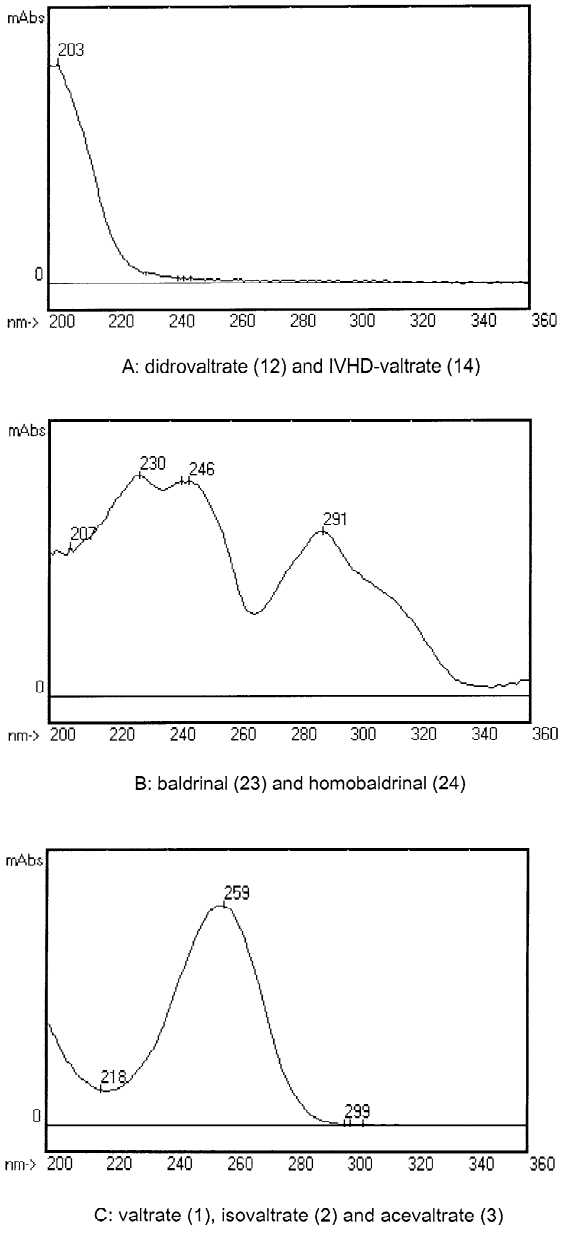



Fig. 10. UV–Vis profile of: (A) didrovaltrate (12) and IVHD-valtrate (14); (B) baldrinal (23) and homobaldrinal (24); (C) valtrate (1), isovaltrate (2) and acevaltrate.

an HPLC method can be chosen for total valepotriate profiling. Despite newly developed methods like CE and *p*-SFC, HPLC methods of analysis remain the first choice for quantitative valepotriate analysis.

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