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Fast, sensitive and selective liquid chromatographic–tandem mass spectrometric determination of tumor-promoting diterpene esters

G. Vogg[a], S. Achatz[b], A. Kettrup[b], H. Sandermann Jr.[a,*]

[a]*Institute of Biochemical Plant Pathology, GSF-National Research Center for Environment and Health, D-85764 Oberschleissheim, Germany*

[b]*Institute of Ecological Chemistry, GSF-National Research Center for Environment and Health, D-85764 Oberschleissheim, Germany*

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Abstract

A liquid chromatography–tandem mass spectrometry (LC–MS–MS) method was developed to detect tumor-promoting diterpene esters of the tigliane and ingenane types within plant extracts. Fractionation on a C_{18} high-performance liquid chromatography (HPLC) column was followed by MS–MS–multiple reaction monitoring (MRM) using the precursor→product ion pairs of m/z 311→293 and 293→265 for phorbol esters. The ion pairs m/z 313→295 and 295→267 were used for ingenol and deoxyphorbol esters. In a second run, the characteristic ions at m/z 311 and 313 were followed in precursor ion scan mode. These quasi-molecular ions were utilized to obtain full scan spectra of the compounds in product ion scan mode. Due to its selectivity, the present on-line method can be applied for plant cultivar selection and plant product control without time-consuming extraction procedures and complex bioassays.

Keywords: *Euphorbia leuconeura*; *Croton* oil; Oils; Plant materials; Diterpenes; Terpenes; Phorbol; Ingenol

1. Introduction

Diterpene esters from *Euphorbiaceae*, like phorbol, deoxyphorbol or ingenol esters, are known to be highly active tumor-promoting agents [1,2]. They do not themselves elicit tumors but promote tumor growth following exposure to a subcarcinogenic dose of a solitary chemical carcinogen. In the classical Berenblum model of co-carcinogenesis [3], a subthreshold dose of a benzo[*a*]pyrene is applied to one group of mice. A similar group receives repeated doses of tumor promoters. Neither of these groups develops tumors. A third group receiving a single subthreshold dose of a carcinogen and repeated submicrogram doses of a tumor promoter develops tumors. Since the report by Castagna et al. [4], it is known that protein kinase C is the primary receptor for this class of compounds. The enzyme activity is highly activated by most of the diterpene derivatives mentioned above. Phorbol-12-tetradecanoate-13-acetate (also known as TPA), which was isolated by Hecker [5] from the latex of *Croton tiglium*, became a classical activator of protein kinase C in many studies related to signal transduction. The acute effect of toxic diterpenes is the induction of skin inflammation [6].

*Corresponding author. Tel.: +49-89-3187-2285; fax: +49-89-3187-3383.

E-mail address: sandermann@gsf.de (H. Sandermann Jr.)

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Because of their adverse effects on human health, fast and selective detection of these diterpene esters is important for the development of less toxic plant cultivars, or food and medicinal preparations. However, due to their lack of characteristic chromophores, the detection of these compounds is often based on sophisticated bioassay-guided fractionations. For example, the irritancy or the tumor-promoting effect on mouse skin is employed. Besides mouse skin experiments, the induction of Epstein-Barr virus in human lymphocytes is a common bioassay [7,8]. In some publications, the molluscicidal effect of these substances was used [9]. The purity of isolated compounds has been monitored by means of analytical thin-layer chromatography (TLC), gas–liquid chromatography (GLC) or mass spectrometry. The toxic diterpene esters are very sensitive towards heat and light, so careful and gentle methods of extraction, separation and detection are necessary. The on-line combination of high-performance liquid chromatography (HPLC) with electrospray ionization tandem mass spectrometry (MS–MS) is becoming increasingly popular as an effective and convenient method for the detection of labile compounds in complex biological matrices.

In this publication, we describe a HPLC–MS–MS method, which is proved here to be selective for these compounds. Under the mild conditions of electrospray ionization, the diterpene esters are transformed to their quasi-molecular ions (adducts of H^+, NH_4^+, Na^+ etc.). Thermal degradation of the compounds could not be observed with this method.

We use tandem mass spectrometry in the MRM mode (multiple reaction monitoring) with collision-induced dissociation. In this mode, a characteristic precursor ion is monitored simultaneously with one of its fragmentation products. As both mass selective quadrupoles are set to a fixed mass, high selectivity and sensitivity (due to reduced chemical noise) is achieved. In most cases, a single extraction with methanol was sufficient as clean-up for HPLC–MS–MS analyses. Subsequent to the selective detection of the diterpene esters, a precursor ion analysis is carried out to identify their molecular masses. Finally, in a third run, an analysis of the product ions of the quasi-molecular ions can be carried out to obtain full scan mass spectra of the investigated compounds.

The present methodology could find practical application in screening for plant cultivars of the spurge family with a reduced content of diterpene esters. This aims to protect gardeners and customers who may get contaminated with latex. Also, food and medicinal herbs of this plant family can be checked for these secondary metabolites.

The described method is illustrated with three examples: Example 1 shows the separation of seven commercially available phorbol and ingenol ester standards. Example 2 is the separation of latex of *Euphorbia leuconeura*, a rare indoor plant from Madagascar with high Epstein Barr virus (EBV)-inducing activity [10]. In example 3, we investigated the phorbol- and deoxyphorbol esters of a commercial *Croton tiglium* seed oil. A similar plant sample has been intensively studied, mainly by Hecker [11]. The constituents are well known, so we were able to use this sample to validate the method presented.

2. Experimental

2.1. Sample preparation

Standard diterpene esters (phorbol-13-acetate, phorbol-12,13-diacetate, phorbol-12,13,20-triacetate, phorbol-12-*N*-methylanthranilate-13-acetate (sapintoxin D), ingenol-3,20-dibenzoate, phorbol-12-tetradecanoate-13-acetate (TPA) and phorbol-12-deoxy-13-tetradecanoate) were purchased from Sigma (Deisenhofen, Germany). The compounds were dissolved in methanol (LiChrosolv, Merck, Darmstadt, Germany) to 10 μg/ml. Prior to MS analysis, the mixture was diluted 1:1 (v/v) with 200 m*M* ammonium acetate (NH_4OAc) to generate NH_4^+ adducts of the compounds.

Latex was collected from *Euphorbia leuconeura* by applying scalpel incisions into the stem and leaves of the plant. White latex was drained into Eppendorf tubes. Subsequently, the samples were weighed and extracted with 1 ml of methanol. The extract was centrifuged for 5 min (16 000 *g*, Centrifuge 5145, Eppendorf, Hamburg, Germany), the supernatant was diluted 1:1 (v/v) with 200 m*M* NH_4OAc and was used for the HPLC separation.

Croton (*tiglium*) oil (Sigma): 10 ml were extracted with four 15 ml portions of 90% aqueous methanol. The methanolic solution was filtered and dried in a

rotary evaporator at 30°C. The yellow residue (resin) was dissolved in 2 ml of methanol, diluted 1:1 (v/v) with 200 m*M* NH_4OAc and used for analyses.

2.2. Instrumentation

The HPLC system consisted of a Waters 600-MS system controller. Mass spectrometry was performed using an API 300 LC–MS–MS system (PE-Sciex, Thornhill, Canada) equipped with an ion spray and a turbo ion-spray interface. LC 2 Tune 1.2 and Multiview1.2 software (PE Sciex) were used for data acquisition and evaluation.

2.3. Column liquid chromatography

A linear water–acetonitrile (Chromosolv, Riedel-de Haën, Seelze, Germany) gradient was used as the mobile phase. The gradient was optimized for different samples in order to achieve sufficient separation of the compounds. The applied gradient was from 0 to 88% acetonitrile over 25 to 60 min (depending on the sample), with a flow-rate of 0.8 ml/min at room temperature. A C_{18} reversed-phase cartridge column (250×4 mm I.D., Supersphere RP 18, not end-capped, 4 μm, Merck) was used as the stationary phase. The injected sample volume was 20 μl. Compounds were detected with a UV detector (Merck–Hitachi, L-7400, Darmstadt, Germany) at a wavelength of 220 nm prior to MS.

2.4. Mass spectrometric conditions

The mass spectrometer was interfaced to the HPLC system via a Turbo Ionspray interface that was maintained at 400°C. The flow of the column effluent was split by 50%. Nitrogen 5.0 (Linde, Höllriegelskreuth,Germany) was used as the nebulizer (1.31 l/min), curtain (1.25 l/min) and collision (pressure in the collision cell $P_{CC}=0.44$ Pa) gas, to effect collision-induced dissociation in the MS–MS mode.

The spectra were obtained by applying an ion spray voltage of 5.0 kV to the column effluent in the positive mode. For the identification procedure, we applied different parameters, which were optimized to obtain maximum intensity of the requested ions. The parameters are summarized in Table 1.

In **Step 1,** the 'prescan run', the parameters were adjusted to achieve maximum intensity of four precursor-to-product ion pairs in the MRM mode: [*m*/*z*] 313→295, 311→293, 295→267, 293→265. Ionization parameters were adjusted to induce in-source fragmentation processes in order to achieve

Table 1
Instrument parameters of the MS–MS system during the three steps of diterpene ester identification[a]

	Step 1 'prescan run'	Step 2 'identification run'	Step 3 'product ion scan'
Ion source (interface)	Turbo Ionspray	Turbo Ionspray	Ionspray
Polarity	Positive	Positive	Positive
Mode	*MRM mode*	*Precursor ion scan*	*Product ion scan*
IS (V)	5000	4800	4800
Temperature (°C)	400	400	25
OR (V)	8	5	5
RNG (V)	282	258	258
Q0 (V)	−18	−8	−8
IQ1 (V)	−18.4	−9	−9
ST (V)	−24	−17.2	−17.2
RO1 (V)	−19.2	−9.2	−9.2
IQ2 (V)	−27	−22	−22
RO2 (V)	−38	−26	−20 (−30)
IQ3 (V)	−68	−44	−50 (−60)
RO3 (V)	−43	−28.5	−25 (−35)
Step size (*u*)		0.25	0.25
Dwell time (ms/step)	500	2	2

[a] In brackets, alternative voltages are given for additional information.

the formation of uniform ions that were characteristic for the skeletons of this compound class (m/z 311 and 313). These fragment ions served as precursor ions in a subsequent MS–MS process (quasi-MS–MS–MS). Instrument parameters were optimized to achieve maximum intensity of the selected precursor ions and their fragment products (Table 1). A signal in this run is a strong indication for the presence of diterpene esters. A distinction between phorbol- and ingenol esters is already possible. Esters of deoxyphorbol, which is an isomer of ingenol, behave like the ingenols.

Step 2, the 'identification run', was carried out in the precursor ion scan mode to determine the 'origin' of the particular product ions, m/z 311 and 313, which are formed (in contrast to step 1) in the collision cell Q2. Therefore, Q1 sweeps a range of m/z 350 to 850, while Q3 is fixed to one of the two product ions.

After this run, either the protonated molecule or the ammonium adduct of the compounds can be identified. With the data achieved in the 'identification run' a 'product ion scan' of the quasi molecular ions can be done (=**Step 3)**. This experiment is carried out in the common MS–MS product ion mode. Q1 is fixed to the particular parent ion, Q3 sweeps a given mass range. This third step is used for gathering structural information and for further identification of the compounds.

Steps 1 and 2 were generally carried out using the Turbo Ionspray interface, which was coupled to the HPLC system. In step 3, it was changed to a direct sample infusion system without chromatographic separation (Harvard Apparatus, Quebec, Canada). With the syringe pump, low flow-rates (5 μl/min) can be adjusted to use the ion-spray interface.

3. Results and discussion

Our main interest was in establishing a fast, sensitive and selective method to detect diterpene

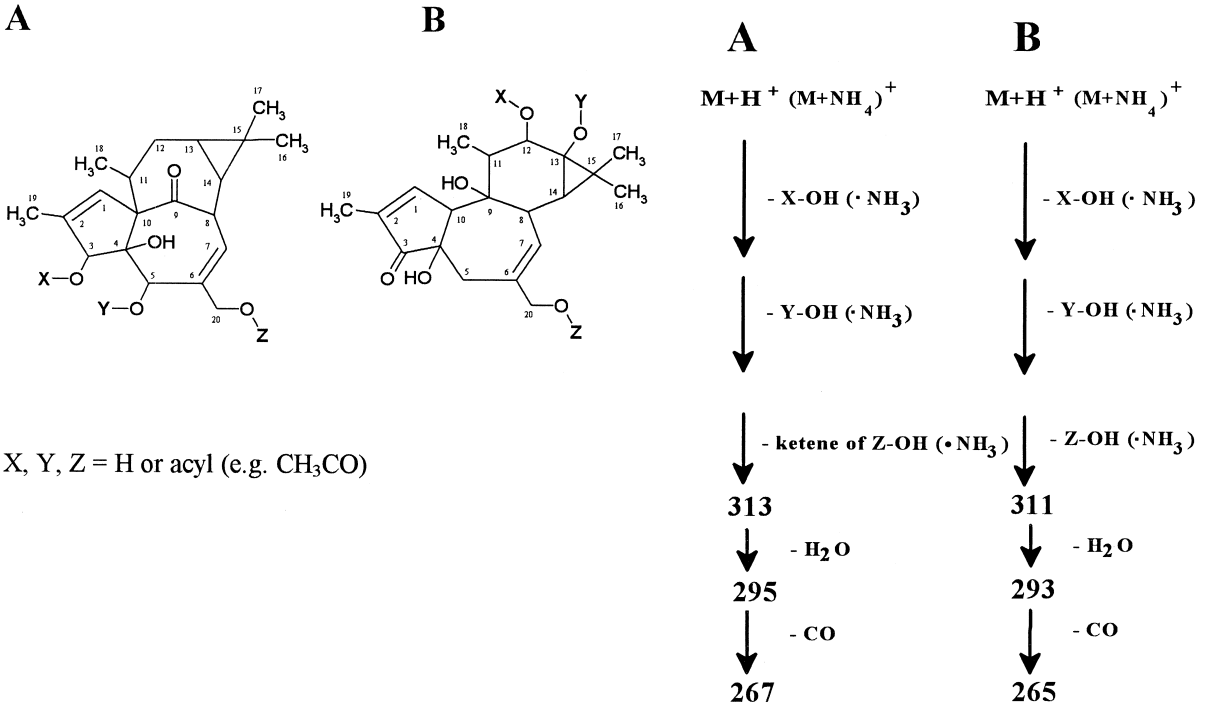

Fig. 1. Principle of fragmentation: Diterpene esters of the ingenane (A) and the tigliane type (B) are fragmented by eliminating their ester groups as free acids (X–OH), resulting in a diterpene nucleus of m/z 313 and 311, respectively. Starting from these skeletons, they further fragment by losing H_2O (−18 u) and CO (−28 u). This characteristic pattern can be used to establish specific detection of these compounds.

esters of the tigliane and ingenane type in different matrices. In contrast to the bioassay-guided isolation, the analytical approach gives an indication also of 'cyptic' and biologically inactive compounds. In LC–MS experiments with phorbol- and ingenol standards, or with isolated diterpene esters from plant sources, we determined a characteristic fragmentation pattern (Fig. 1). Different phorbol esters led to protonated molecules and/or ammonium adducts. During fragmentation, the loss of three molecules of water or organic acid (RCOOH) was accompanied by loss of ammonia, thus resulting in a protonated phorbol nucleus, m/z 311 ($C_{20}O_3H_{23}$). A similar behavior was observed for ingenol and the isomeric deoxyphorbol esters. However, in these cases, the obtained ingenol/deoxyphorbol nucleus appeared at m/z 313 ($C_{20}O_3H_{25}$), which is 18 u (H_2O) higher than the analogous phorbol signal. This agrees with the different number of hydroxy groups in the free diterpene alcohols. While phorbol exhibits five OH groups, of which three can be eliminated to give a stable fragment ion, ingenol/deoxyphorbol possess only four. To obtain a similarly stable fragment ion, only two ester/hydroxy groups are eliminated as free acid or water. The third elimination step, according to Evans and Taylor [12], occurs via loss of a ketene ($H_2C{=}CO$). These diterpene skeletons underwent a loss of H_2O (-18 u).

This fragmentation could also be caused by collision-induced dissociation in the MS–MS mode. Therefore, we chose the pair of fragments m/z 311→293 in MS–MS mode to examine selectively for phorbol esters and m/z 313→295 for ingenol and the isomeric deoxyphorbol esters. In addition, but to a lower extent, a characteristic elimination of 28 u ($-CO$) from the fragments m/z 295 and 293 could be observed. Therefore, we measured the pairs m/z 293→265 for phorbol- and m/z 295→267 for ingenol esters as an additional confirmation of the presence of these diterpenes in unknown samples.

In Example 1, seven different phorbol-/deoxyphorbol and ingenol esters were separated and examined with the method described. The chromatogram is presented in Fig. 2. In this run we applied 100 ng per standard. No distinction could be achieved between the isomeric ingenol- and deoxyphorbol skeletons. The fragment pairs m/z 293→265 and m/z 295→267 could be used for further confirmation of the diterpene esters (Fig. 2C–E). However, it was less sensitive and its intensities were discriminating between the different compounds.

In a second run (in the precursor ion scan mode), we used the ion current at m/z 311 and 313, respectively, to identify their precursor ions (Figs. 3 and 4). In general, in order to obtain the ammonium adducts, the samples were diluted 1:1 (v/v) with NH_4OAc prior to analysis. In the final step, the quasi-molecular ions could be used to record full-scan spectra (product ion scan mode) of the compounds (data not shown). These spectra provide

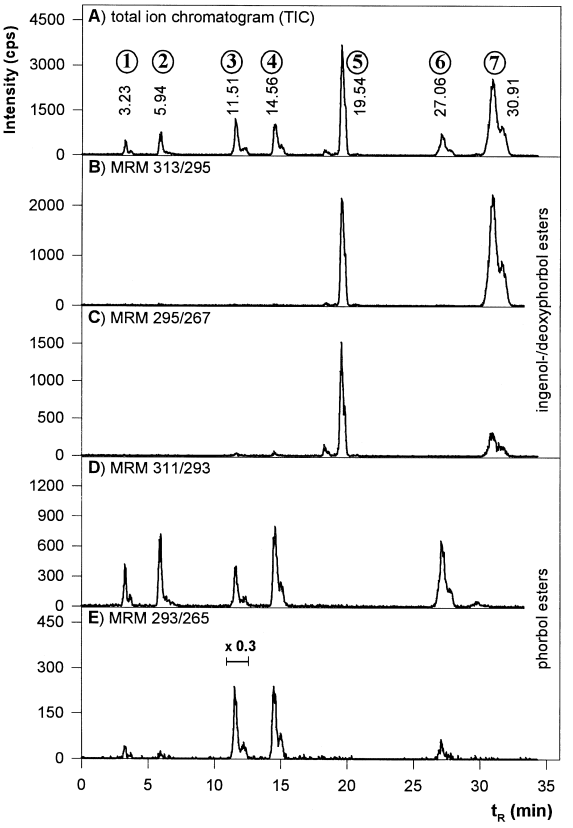

Fig. 2. 'Prescan run' of a standard mixture of seven diterpene esters (cps=counts per second; t_R=retention time). (A) total ion chromatogram, (B) chromatogram of m/z 313→295 (ingenol- and deoxyphorbol esters), (C) chromatogram of m/z 295→267 (confirmation of ingenol- and deoxyphorbol esters), (D) chromatogram of m/z 311→293 (phorbol esters) and (E) chromatogram of m/z 293→265 (confirmation of phorbol esters).

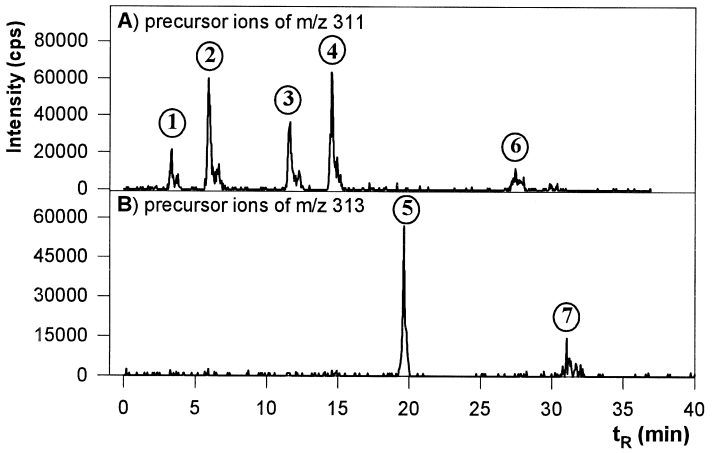

Fig. 3. 'Identification run' of a standard mixture of seven diterpene esters. (A) Precursor ion scan of m/z 311, to detect phorbol esters. (B) Precursor ion scan of m/z 313, to detect ingenol- and deoxyphorbol esters.

Table 2
Phorbol and deoxyphorbol derivatives identified from commercial *Croton tiglium* seed oil. The methanolic extract was analyzed with the three steps of the LC–MS–MS method, as described in the Experimental section

Compound	$[M+H]^+$ (u)	Ref.
Deoxyphorbol acetate	391	
Phorbol acetate	407	
Phorbol methylbutenoate	447	
Phorbol diacetate	449	
Deoxyphorbol acetate methylbutenoate	473	[14]
Phorbol butanoate acetate	477	
Phorbol acetate methylbutenoate	489	[14]
Phorbol triacetate	491	
Deoxyphorbol methylbutanoate methylbutenoate	515	
Phorbol butanoate methylbutenoate	499	
Deoxyphorbol butanoate methylbutenoate	501	
Phorbol butanoate methylbutenoate	517	[13,14]
Phorbol butanoate methylbutanoate	519	
Deoxyphorbol diacetate hexadienoate or deoxyphorbol acetate nonadienoate	545	
Phorbol diacetate methylbutenoate	531	
Phorbol octanoate acetate	533	
Phorbol acetate butanoate methylbutenoate	559	
Phorbol acetate decanoate	561	[13,14]
Phorbol octanoate methylbutenoate	573	[13]
Phorbol octanoate methylbutanoate	575	[13]
Phorbol dodecanoate acetate	589	[13]
Phorbol decanoate methylbutenoate	601	[13]
Phorbol decanoate methylbutanoate	603	[13]
Phorbol undecanoate methylbutenoate	615	
Phorbol tetradecanoate acetate	617	[13,14]
Phorbol dodecanoate methylbutenoate	629	[13]
Phorbol hexadecanoate acetate or phorbol diacetate tetradecanoate	645	[13]
Phorbol nonadecanoate acetate	685	
Phorbol hexadecanoate methylbutenoate	687	

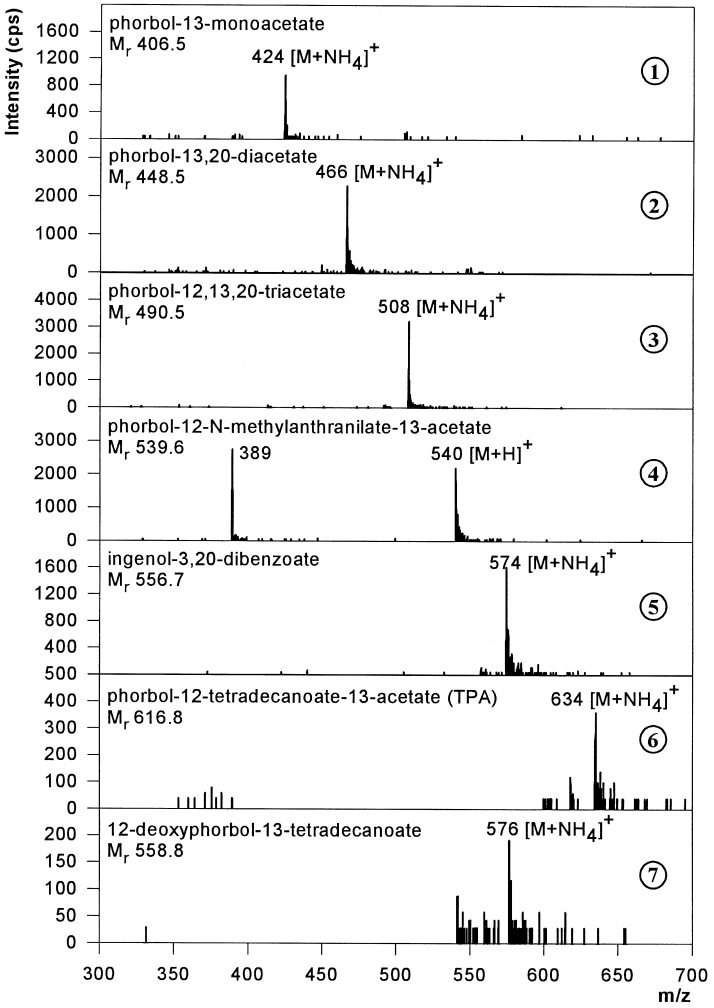

Fig. 4. Precursor ion spectra of peaks 1 to 7 (Figs. 2 and 3). Due to the presence of 100 m*M* NH_4OAc in the sample, in most cases, the ammonia adducts of the molecular masses were achieved.

further evidence for the presence of diterpene esters, due to their characteristic fragmentation pattern.

In Example 2, we show an analysis of the milky latex of *Euphorbia leuconeura*. Because of its attractive leaves, it is an occasionally used indoor plant. The milky latex possesses high EBV-inducing activity [10]. Three ingenol esters were isolated and characterized. The isolation was carried out by fractionation of the latex extract, which was guided by determining EBV-inducing activity of each single fraction. The 'prescan' step (Fig. 5A) detected three distinct ingenol esters within the latex. A small signal could also be measured at 10.3 min. No signal was visible in the m/z 311→293 ('phorbol-') trace. In the 'identification' step (313 ← quasi-molecular ion) (Figs. 5B and C), signals derived from two isomeric compounds (NH_4^+-adducts) with molecular masses of 629 and one with 749 were detected. These results agree with previous data [10]. In Fig. 5D, the product ion spectrum of m/z 629 is presented. The ingenol esters of this extract belong to the group of the milliamines, with a characteristic aromatic peptidyl residue as one of the ester groups (Fig. 5E). It also became obvious that the small signal at 10.3 min derived from ingenol monoacetate, which seems to be a decomposition product of the

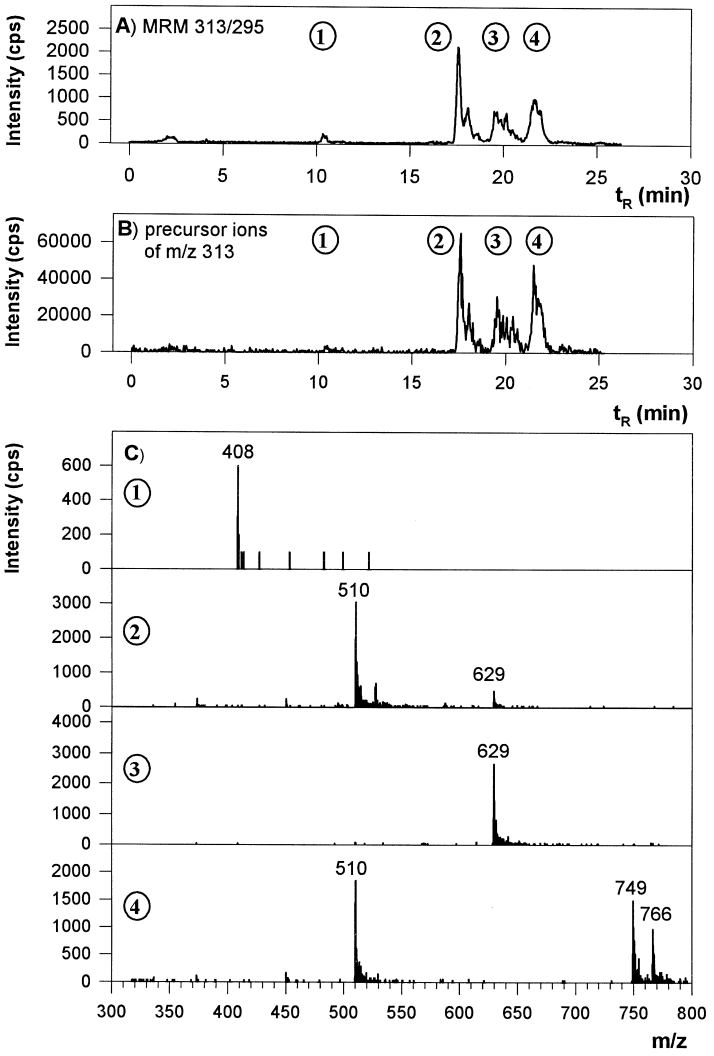

Fig. 5. (A) 'Prescan run' of a methanolic extract of latex from *Euphorbia leuconeura*. MRM of m/z 313→295, to detect ingenol and deoxyphorbol esters. (B) 'Identification run': precursor ion scan of m/z 313, to identify the molecular masses of ingenol- or deoxyphorbol esters. (C) Precursor ion spectra of peaks 1 to 4 (A and B). (D) MS–MS product ion scan of m/z 629. (* Intensity multiplied by 0.5). (E) Chemical structure of peak 2 (Fig. 5D).

milliamines. Its intensity increased with increasing storage time of the sample, even at −20°C.

In Example 3, *Croton* (*tiglium*) seed oil was investigated for phorbol and deoxyphorbol esters. Hecker and Schmidt [13] and Bauer et al. [14] had previously identified 19 different diterpene esters from this plant source. The chromatograms of the 'prescan runs' of m/z 311→293 and 313→295 of a methanolic raw extract of *Croton* oil are shown in Fig. 6A and B. The corresponding chromatogram of the 'identification runs' show the same signal pattern (data not shown). From these chromatograms, the presence of at least 30 different diterpene esters could be assumed. The third step (product ion scans) led to a list of phorbol and deoxyphorbol esters, which was deduced from the mass spectrometric data (Table 2).

Twenty-nine different phorbol and deoxyphorbol

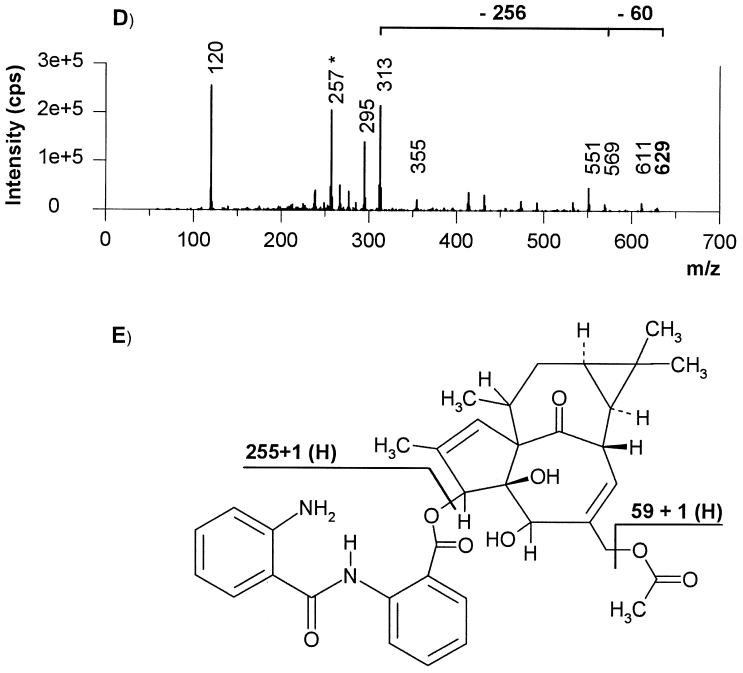

Fig. 5. (*continued*)

esters could be identified from *Croton* oil with this on-line detection method. Fig. 6C shows the mass spectrum of TPA, identified from this extract (Fig. 6D). To date, 19 of the listed compounds have been characterized [13–15]. The presence of further phorbol- and deoxyphorbol esters, which have not been described yet, is likely. Phorbol-dodecanoate-tigliate was described as the product of partial hydrolysis [13]. With our LC–MS–MS method, it could be shown that this compound is present in the *Croton* oil. However, the separation of the raw extract by HPLC made it difficult to distinguish between all of the diterpene compounds. Therefore, certain diterpene esters that had been published previously could not be identified (e.g. phorbol linoleate methylbutenoate acetate [14], phorbol methylbutanoate dodecanoate [13] or phorbol butanoate dodecanoate [13]).

4. Conclusion

It could be shown that coupling of HPLC and MS–MS offers a specific and sensitive method to detect diterpene esters of the tigliane and ingenane type. In three examples, we demonstrated that our method works even with complex and difficult matrices. Therefore, it seems suitable for the large-scale screening of biological materials of different origins. The three steps for the identification of these compounds are independent of each other and, thus, can be applied as a modular system. Screening of biological samples for the presence of toxic diterpene esters only requires the very sensitive ‘prescan’ step.

If the existence of diterpene esters in a sample is already known, further structural information can be achieved with the ‘identification’ step and the subsequent product ion scan. Practical applications in the selection of plant cultivars (e.g. *Euphorbiaceae* indoor plants) and in the control of food or medicinal herbal preparations are envisaged.

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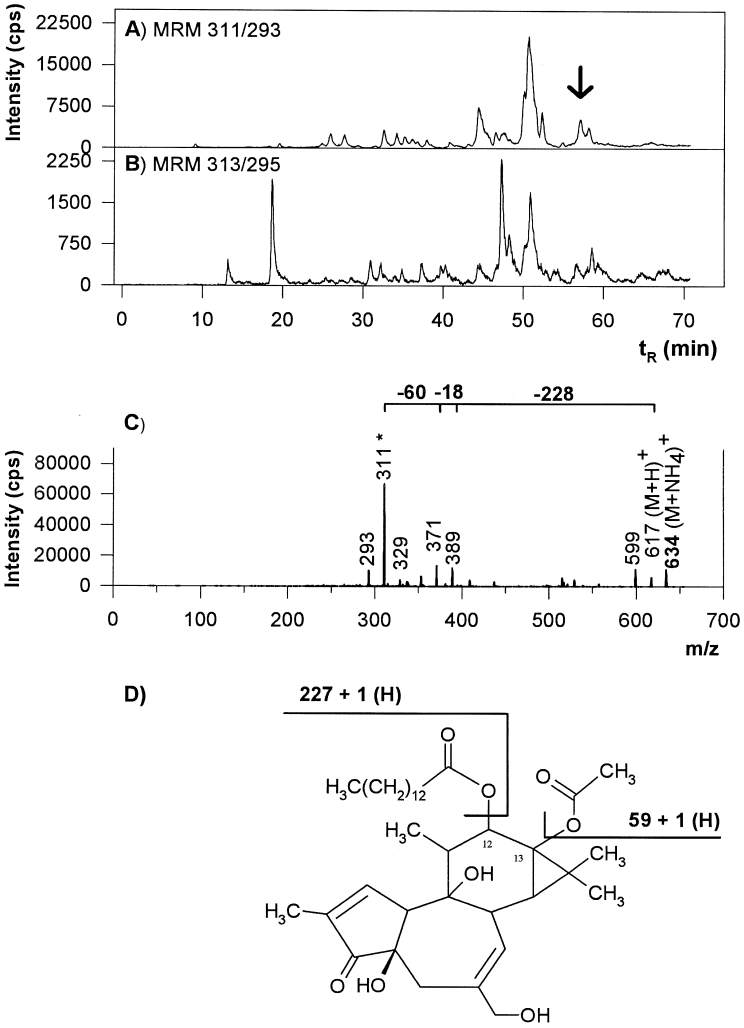

Fig. 6. 'Prescan run' of *Croton* oil extract: (A) MRM 311/293 (phorbol esters) and (B) MRM 313/295 (ingenol and deoxyphorbol esters). Marked peak is coming from *m*/*z* 634 (precursor ion scan not shown). (C) MS–MS product ion scan of *m*/*z* 634 from the *Croton* oil extract. (* Intensity multiplied by 0.5). (D) Structure of marked peak (*m*/*z* 634): phorbol-12-tetradecanoate-13-acetate (TPA).

(Chappes, France) and by Bayerisches Staatsministerium für Landesentwicklung und Umweltfragen (Munich, Germany).

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