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Determination of the Absolute Configuration of 6-Alkylated α-Pyrones from *Ravensara crassifolia* by LC-NMR

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The absolute configuration of asymmetric centres of two α-pyrones isolated from *Ravensara crassifolia* was determined using the Mosher method. The conventional analysis of the purified ester derivatives by ^{1}H-NMR was replaced by a rapid and sensitive method in which the α-pyrones were analysed under isocratic reversed-phase LC-NMR conditions prior to and after derivatisation reactions. Comparison of the LC-^{1}H-NMR spectra of the actual α-pyrones with those of the corresponding Mosher's esters recorded in the acetonitrile:deuterated water solvent system exhibited shifts comparable with those obtained using conventional deuterated solvents. Based on the shifts recorded, determination of the absolute configuration was possible by application of Mosher rules. The use of LC-NMR has permitted a direct analysis of crude reaction mixtures containing less than 50 μg of the starting material. Completion of the reaction was checked by LC-MS and the crude reaction mixture was analysed by stop-flow LC-NMR. This methodology seems very promising for the determination at the micro-scale level of the absolute configuration of natural products which are available only in very small amounts. Copyright © 2003 John Wiley & Sons, Ltd.

Keywords: LC-NMR; LC-MS; absolute configuration; Mosher's esters; α-pyrones; *Ravensara crassifolia*.

INTRODUCTION

Determination of absolute configuration remains a challenging task in the structure elucidation of natural products. Only a few physical methods, such as chirality excitation (Harada and Nakanishi, 1983) and X-ray crystallography (Fiud *et al.*, 1977) provide the required information, but they have some limitations. A chemical method involving the synthesis of Mosher's esters has been frequently used for the characterisation of various natural products bearing secondary alcohol functions (Ohtani *et al.*, 1991; Gu *et al.*, 1994; Jeong *et al.*, 2000; Rasmussen *et al.*, 2000). In this case, the H^1-NMR spectra of (*R*)- and (*S*)-2-methoxy-2-phenyl-(trifluoromethyl) acetic acid (MTPA) ester derivatives of the analytes are compared. The difference in chemical shifts of the diastereoisomers determined from:

$$\Delta\delta_H = \delta_S - \delta_R \quad (1)$$

indicates whether the alcohol is (*R*) or (*S*) based on established conformational models (Dale and Mosher, 1968, 1973; Dale *et al.*, 1969; Sullivan *et al.*, 1973; Trost *et al.*, 1986). Although it is a very efficient method for determining absolute configuration, the synthesis of Mosher's esters requires relatively large amounts of samples for derivatisation. When working with natural products, pure compounds are often available in only very limited amounts and this hinders the determination of absolute configuration by conventional methods.

With the recent development of LC-NMR, analysis of the ^{1}H-NMR of natural products in the microgram range has become possible. This technique also allows the chromatographic resolution of an LC-peak of interest prior to NMR detection (Wolfender *et al.*, 2001). Taking advantage of this new hyphenated LC technique, a means of allowing the direct analysis of the crude Mosher's ester reaction mixtures has been developed and validated with model compounds of known absolute configuration. The aim of the study was to establish a method for derivatisation and subsequent rapid determination of the absolute configuration using only a few μg of natural products.

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EXPERIMENTAL

Preparation and purification of Mosher's esters. 6*S*-(2*S*-Hydroxy-6-phenylhexyl)-5,6-dihydro-2*H*-pyran-2-one (**1**; 5 mg in 2 mL of dichloromethane) was sequentially treated with pyridine (0.2 mL) and (*R*)-(−)-MTPA chloride (100 mg). The mixture was stirred at room temperature under an atmosphere of nitrogen for 5 h, and the progress of the reaction was monitored by HPLC. The reaction mixture was concentrated and dried, and the residue dissolved in dichloromethane and washed with 1% sodium bicarbonate (5 mL) and water (2 × 5 mL). The organic layer was purified by HPLC using a Waters (Milford, MA, USA) Bondapak® C_{18} column (100 × 25 mm i.d.; 10 μm) affording (*S*)-Mosher's ester derivative (**1a**) (5 mg; 56% yield). The (*R*)-Mosher's ester derivative (**1b**) (6 mg; 67.1% yield) was prepared using (*S*)-(+)-MTPA chloride under the conditions described above. Mosher's ester derivatives of 6*R*-(4*S*,6*S*-dihydroxy-10-phenyl-1-decenyl)-5,6-dihydro-

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2*H*-pyran-2-one (**2**), i.e. compounds **2a** and **2b**, were prepared in an exactly similar fashion.

Preparation of Mosher's ester by micro-reaction. Compound **1** (50 μg in 100 μL of dichloromethane) was transferred to a 2 mL HPLC vial with a capped septum and treated with 20 μL of pyridine and 100 mg of (*R*)-(−)-MTPA chloride. This low amount of pyridine compared with MTPA chloride was found not to affect the reaction yield. The mixture was stirred at room temperature under an atmosphere of nitrogen for 4 h and the progress of the reaction was monitored by LC-UV-APCI/MS (10 μL of the reaction mixture was analysed, being 5% of the total reaction mixture). Dichloromethane was removed with nitrogen flux, and the total mixture was solubilised in 100 μL of acetonitrile (HPLC grade) and analysed by stop-flow LC-NMR [the number of transients (NT) was 1024]. Mosher's ester derivative (**1b**) was prepared using (*S*)-(+)-MTPA chloride under the conditions described above.

LC-UV-APCI/MS analysis. Reversed-phase HPLC analysis of compounds **1, 1a, 1b, 2, 2a** and **2b** was performed on a Hewlett-Packard (Waldbronn, Germany) series 1100 liquid chromatograph system with photodiode array detector (PAD), connected in series to a Finnigan MAT (San Jose, CA, USA) LCQ ion trap mass spectrometer. Compounds (250 μg of each) were injected onto a Bondapak® C_{18} column (100 × 8 mm i.d.; 10 μm) which was eluted with acetonitrile:water (80:20) for 35 min at a flow rate of 0.8 mL/min. UV traces were recorded at 210 and 254 nm and PAD spectra were recorded between 200 and 500 nm. LC-MS, which was performed directly after UV-PAD analysis, employed an APCI interface with the following conditions: operation, positive mode; capillary temperature, 150°C; vaporizer temperature, 70°C; sheath gas flow, 60; corona needle current, 5 μA; spectral range, 150–900 mu).

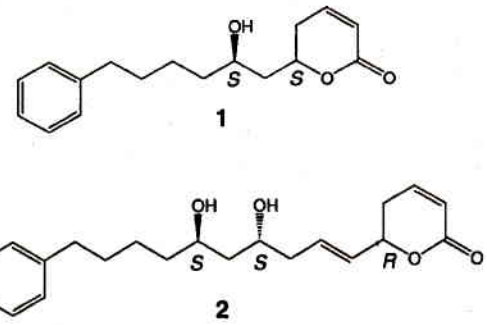

Figure 1. α-Pyrones, 6*S*-(2*S*-hydroxy-6-phenylhexyl)-5,6-dihydro-2*H*-pyran-2-one (**1**) and 6*R*-(4*S*,6*S*-dihydroxy-10-phenyl-1-decenyl)-5,6-dihydro-2*H*-pyran-2-one (**2**), isolated from the aerial parts of *Ravensara crassifolia*.

LC-NMR analysis. A Varian (Palo Alto, CA, USA) Unity Inova 500 MHz NMR instrument equipped with a $^{1}H[^{13}C]$ pulse field gradient indirect detection microflow LC-NMR probe (flow cell, 60 μL, 3 mm i.d.) was used. Reversed-phase HPLC analysis of the compounds was carried out on a Varian modular HPLC system compris-

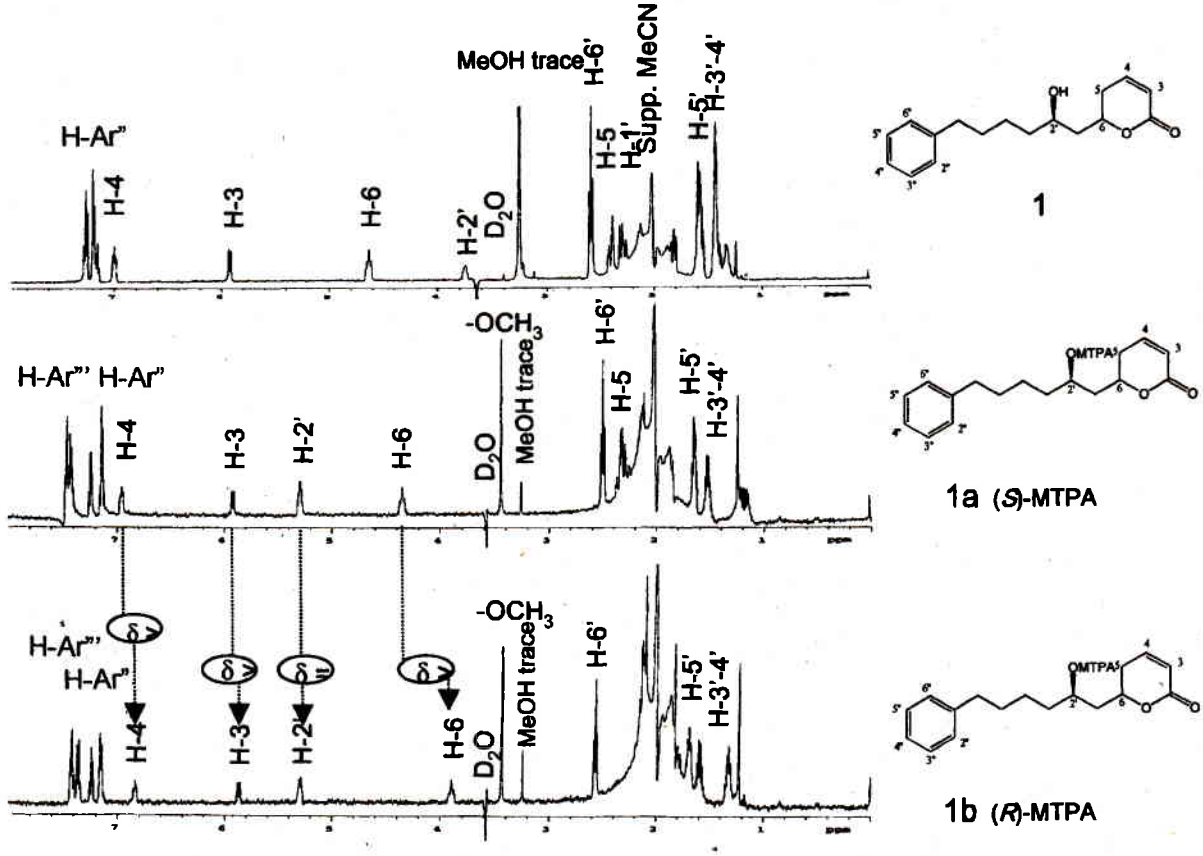

Figure 2. Stop-flow LC-NMR spectrum of **1** and of the Mosher's esters **1a** and **1b** (sample size 250 μg; NT = 128). (For analytical conditions see Experimental section.)

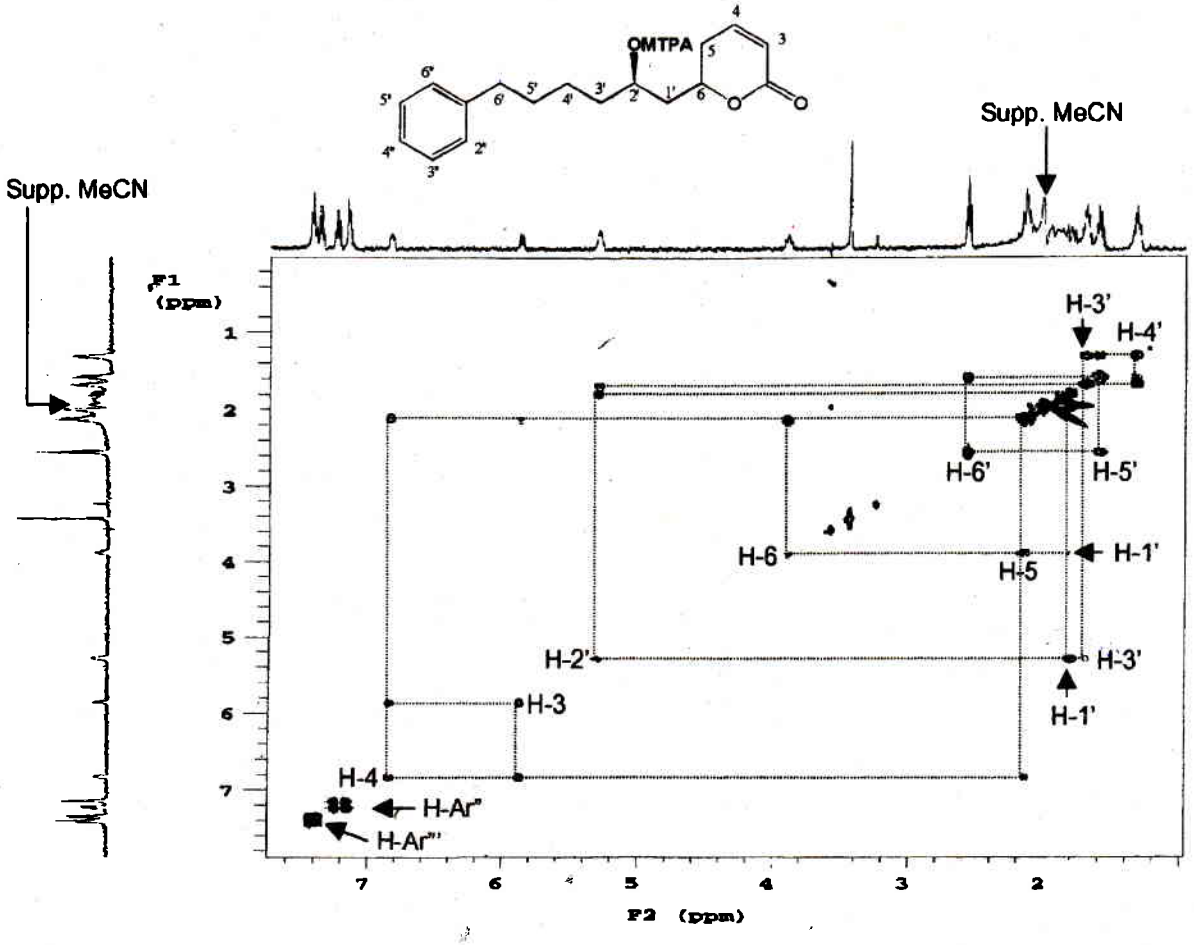

Figure 3. Stop-flow LC-NMR WETGCOSY spectrum of **1b** (sample size 250 μg; NI = 512; NT = 36). (For analytical conditions see Experimental section.)

ing a model 9012 pump, a model 9050 UV detector and a Valco injection valve. The separation was performed using the same conditions as given above for LC-MS analysis except that deuterated water was employed in the mobile phase, and UV traces were measured only at 210 nm. The reference of the solvent signal was set at δ 2.00 for acetonitrile, and for analysis involving samples sizes of 250 μg NT was set to 128. The LC-NMR COSY experiment was performed using a WETGCOSY sequence (NI = 512; NT = 36). For the reaction mixture

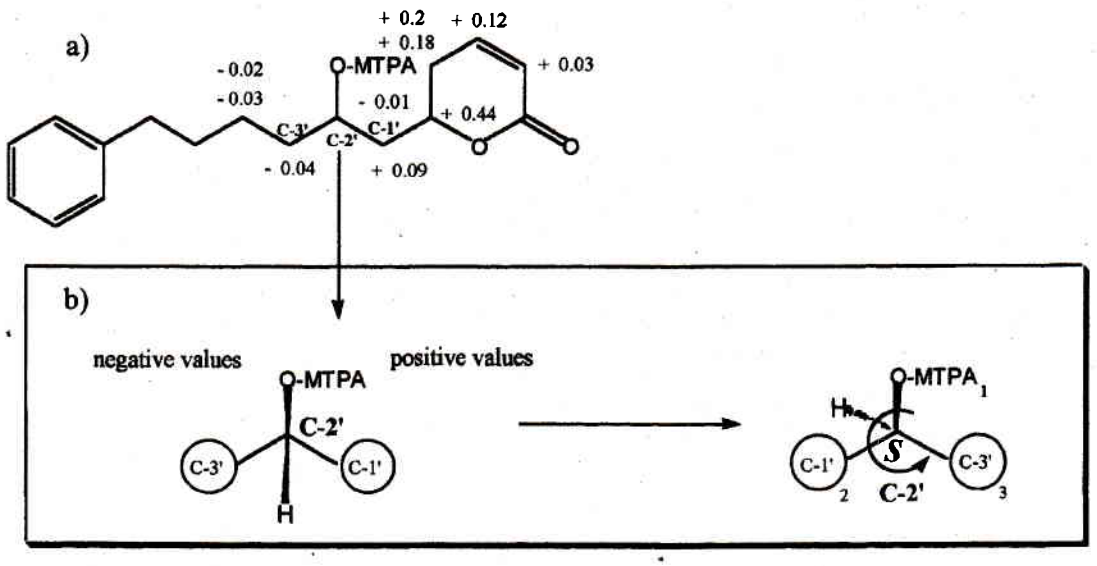

Figure 4. Determination of the absolute configuration with $\Delta\delta_H$ values [obtained using equation (1)] of compounds **1a** and **1b** using the Mosher method.

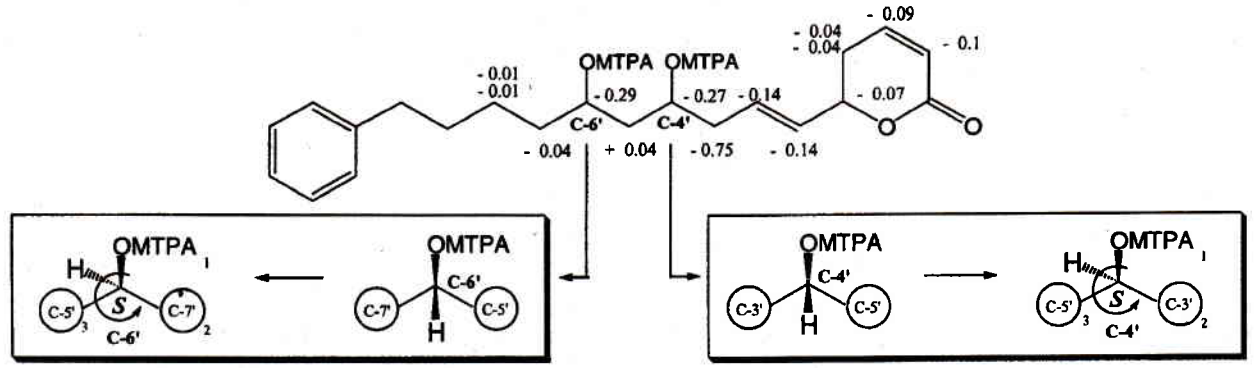

Figure 5. Determination of the absolute configuration with $\Delta\delta_H$ values [obtained using equation (1)] of compounds **2a** and **2b** using the Mosher method.

(50 μg) a good LC-^{1}H-NMR spectra was obtained using NT = 1024.

LC-MS and LC-NMR data of 1, 1a, 1b, 2, 2a and 2b

6*S*-(2*S*-Hydroxy-6-phenylhexyl)-5,6-dihydro-2H-pyran-2-one (1)

^{1}H-NMR (in $CDCl_3$): see Raoelison *et al.* (2001). LC-^{1}H-NMR stop-flow mode: δ 5.94 (1H, d, J = 9.7 Hz, H-3), 7.02 (1H, m, H-4), 2.28 (2H, m, H-5), 4.65 (1H, m, H-6), 1.65–1.83 (2H, H-1′), 3.75 (1H, m, H-2′), 1.47 (2H, H-3′), 1.38–1.48 (2H, H-4′), 1.25–1.52 (2H, H-5′), 2.61 (2H, t, J = 9.7 Hz, H-6′), 7.16–7.25 (H-Ar″, H-2″, 3″, 4″, 5″, 6″). LC-MS APCI-positive ion mode [*m/z* (relative intensity)]: 275 $[M + H]^+$ (45), 239 (50), 221.2 (92), 211.1 (100), 171.3 (63).

8-(*S*)-MTPA ester of 1 (1a)

LC-^{1}H-NMR stop-flow mode (250 μg with NT = 128, or 50 μg with NT = 1024), (reaction mixture)): δ 5.92 (1H, d, J = 9.7 Hz, H-3), 6.96 (1H, m, H-4), 2.31–2.33 (2H, m, H-5), 4.35 (1H, m, H-6), 1.89–2.00 (2H, H-1′), 5.29 (1H, m, H-2′), 1.65 (2H, m, H-3′), 1.30–1.60 (2H, H-4′), 1.25–1.52 (2H, H-5′), 2.58 (2H, t, J = 9.7 Hz, H-6′), 7.16–7.25 (H-Ar″, H-2″, 3″, 4″, 5″, 6″), 3.42 (3H, s, OCH_3-MTPA), 7.20–7.42 (H-Ar‴, phenyl MTPA). LC-MS APCI-positive ion mode [*m/z* (relative intensity)]: 490.8 $[M + H]^+$ (25), 257 (78), 239.2 (100), 221.2 (95), 211.2 (76).

8-(*R*)-MTPA ester of 1 (1b)

LC-^{1}H-NMR stop-flow mode (250 μg with NT = 128, or 50 μg with NT = 1024), (reaction mixture)): δ 5.89 (1H, d, J = 9.7 Hz, H-3), 6.84 (1H, m, H-4), 2.11–2.15 (2H, m, H-5), 3.91 (1H, m, H-6), 1.80–1.91 (2H, H-1′), 5.30 (1H, m, H-2′), 1.69 (2H, m, H-3′), 1.32–1.61 (2H, H-4′), 1.25–1.57 (2H, H-5′), 2.58 (2H, t, J = 9.7 Hz, H-6′), 7.16–7.25 (H-Ar″, H-2″, 3″, 4″, 5″, 6″), 3.42 (3H, s, OCH_3-MTPA), 7.20–7.42 (H-Ar‴, phenyl MTPA). LC-MS APCI-positive ion mode [*m/z* (relative intensity)]: 490.8 $[M + H]^+$ (25), 257 (77), 239.1 (100), 221.2 (93), 211.2 (72).

6*R*-(4*S*,6*S*-Dihydroxy-10-phenyl-1-decenyl)-5,6-dihydro-2H-pyran-2-one (2)

^{1}H-NMR (in $CDCl_3$): see Raoelison *et al.* (2001). LC-^{1}H-NMR (MeCN: D_2O, 80:20) stop-flow mode: δ 603 (1H, d, J = 9.5 Hz, H-3), 6.85 (1H, m, H-4), 2.43 (2H, m, H-5), 4.89 (1H, m, H-6), 5.68 (1H, m, H-1′), 5.85 (1H, m, H-2′), 2.28 (1H, m, H-3′), 3.91 (1H, m, H-4′), 1.58 (1H, m, H-5′), 4.00 (1H, m, H-6′), 1.46–1.57 (1H, H-7′), 1.32–1.44 (1H, H-8′), 1.60 (1H, m, H-9′), 2.67 (1H, t, J = 9.6 Hz, H-10′), 7.12–7.20 (H-Ar″, H-2″, 3″, 4″, 5″, 6″). LC-MS APCI-positive ion mode [*m/z* (relative intensity)]: 344.8 $[M + H]^+$ (100), 309.2 (22), 291.2 (12), 263.2 (), 206.9 (17), 171.4 (28).

4′,6′-Di-(*S*)-MTPA ester of 2 (2a)

LC-^{1}H-NMR stop-flow mode (250 μg with NT = 128): δ 593 (1H, d, J = 9.5 Hz, H-3), 6.94 (1H, m, H-4), 2.33 (2H, m, H-5), 4.81 (1H, m, H-6), 5.57 (2H, m, H-1′ and H-2′), 1.76 (2H, m, H-3′), 4.91 (1H, m, H-4′), 2.47 (2H, m, H-5′), 4.82 (1H, m, H-6′), 2.01 (2H, m, H-7′), 1.26–1.29 (1H, H-8′), 1.52–1.60 (2H, H-9′), 2.59 (2H, t, J = 9.6 Hz, H-10′), 7.12–7.20 (H-Ar″, H-2″, 3″, 4″, 5″, 6″), 3.43 (3H, s, OCH_3, MTPA), 3.50 (3H, s, OCH_3, MTPA), 7.50–7.52 (10H, H-Ar‴, H-Ar⁗, 2 MTPA). LC-MS APCI-positive ion mode [*m/z* (relative intensity)]: 776.8 $[M + H]^+$ (100), 543.1 (22), 309 (12), 291 (10), 223.1 (17).

4′,6′-Di-(*R*)-MTPA ester of 2 (2b)

LC-^{1}H-NMR stop-flow mode (250 μg with NT = 128): δ 603 (1H, d, J = 9.5 Hz, H-3), 7.03 (1H, m, H-4), 2.37 (2H, m, H-5), 4.88 (1H, m, H-6), 5.71 (2H, m, H-1′ and H-2′), 2.51 (2H, m, H-3′), 5.18 (1H, m, H-4′), 2.43 (2H, m, H-5′), 5.11 (1H, m, H-6′), 2.05 (2H, m, H-7′), 1.29–1.34 (1H, H-8′), 1.52–1.60 (2H, H-9), 2.59 (2H, t, J = 9.6 Hz, H-10), 7.12–7.20 (H-Ar″, H-2″, 3″, 4″, 5″, 6″), 3.43 (3H, s, OCH_3, MTPA), 3.50 (3H, s, OCH_3, MTPA), 7.50–7.52 (10H, H-Ar‴, H-Ar⁗, 2 MTPA). LC-MS APCI-positive ion mode [*m/z* (relative intensity)]: 776.8 $[M + H]^+$ (100), 543 (22), 309 (15), 291 (10), 223.3 (17).

RESULTS AND DISCUSSION

In order to evaluate the potential of LC-NMR for the determination of the absolute configuration in the microgram range, two α-pyrones 6*S*-(2*S*-hydroxy-6-phenylhexyl)-5,6-dihydro-2*H*-pyran-2-one (**1**) and 6*R*-(4*S*,6*S*-dihydroxy-10-phenyl-1-decenyl)-5,6-dihydro-

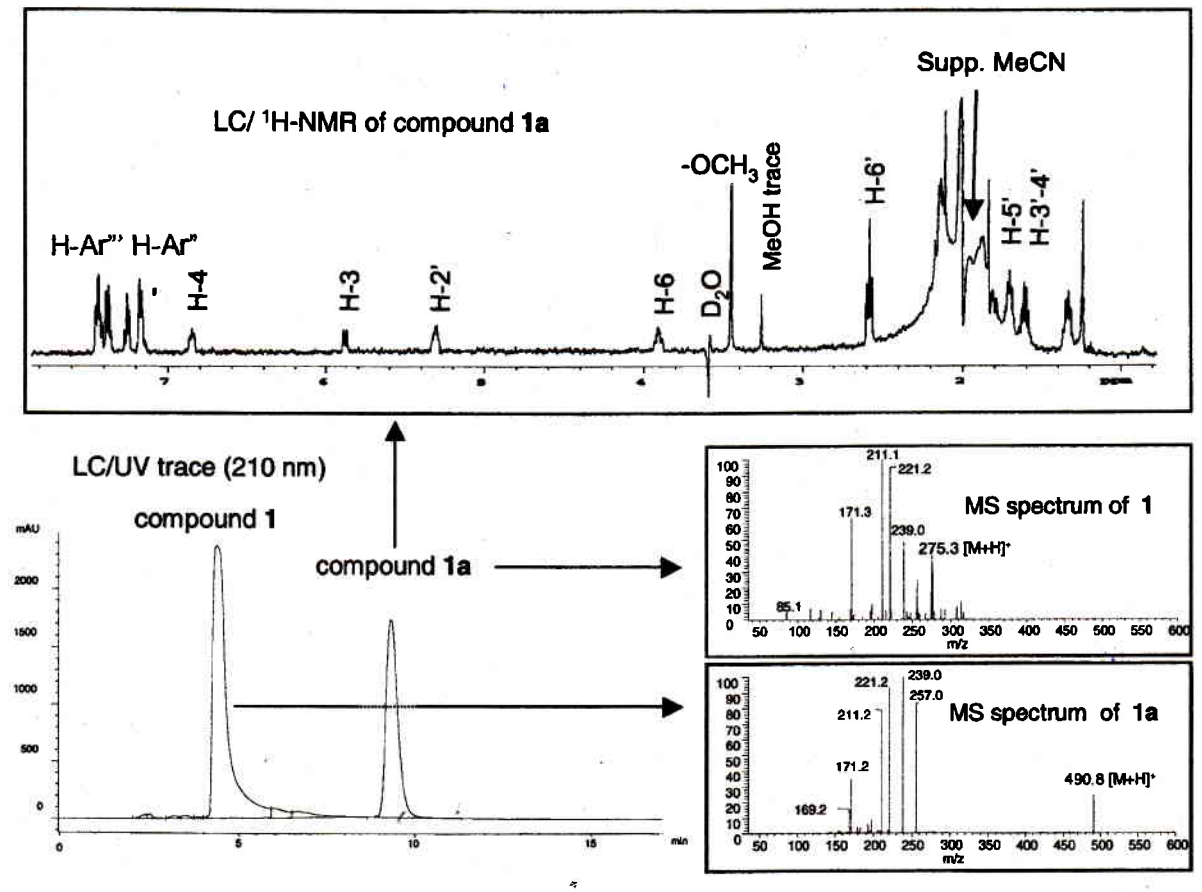

Figure 6. LC-UV-MS and LC-UV-NMR (sample size 50 µg; NT = 1024) analysis of the micro-reaction mixture of compound 1 after 2 h reaction time.

2*H*-pyran-2-one (**2**) (Fig. 1), isolated from the root bark of *Ravensara crassifolia* (Lauraceae), were selected as model compounds (Raoelison *et al.*, 2001, 2002). The classical Mosher reaction was performed on 5 mg of **1** and **2**, which were esterified with (*R*)- and (*S*)-MTPA. The four different ester derivatives (**1a, 1b, 2a** and **2b**) were purified by semi-preparative HPLC and the ^{1}H-NMR spectrum of each purified derivative was recorded in deuterated chloroform. A comparison of the ^{1}H-NMR chemical shifts of the protons adjacent to the esterified hydroxyl group between **1a** (*S*)-MTPA and **1b** (*R*)-MTPA allowed the determination of the absolute configuration of C-2′ to be *S*. In the case of the α-pyrone **2**, a comparison between derivatives **2a** (*S*)-MTPA and **2b** (*R*)-MTPA indicated that the two asymmetric centres at C-4′, C-6′ were *S,S* (Raoelison *et al.*, 2001, 2002).

The LC-NMR analysis of the Mosher's esters was performed using isocratic reversed-phase HPLC conditions with acetonitrile:deuterated water as mobile phase. A C_{18} radial compression column having a large internal diameter (8 mm) was preferred to classical analytical columns because of its high loading capacity. Passage of the crude reaction mixture through the HPLC column separated the Mosher's ester derivatives from the reactants and residual starting material. Isocratic rather than gradient elution was employed since all spectra needed to be recorded in the same solvent for comparison purposes. Under these isocratic LC-NMR conditions, 250 µg of α-pyrone **1** and its purified (*R*)- and (*S*)-MTPA derivatives were analysed by stop-flow LC-NMR. This mode of operation was preferred to on-flow since it allows the acquisition of a higher number of transients and permits two-dimensional correlation experiments such as ^{1}H-^{1}H WET COSY to be conducted. Solvent suppression was achieved using the "water suppression enhanced through T_1 effects" (WET) technique (Smallcombe *et al.*, 1995), which is both fast and reliable.

Good-quality LC-^{1}H-NMR spectra were recorded for all derivatives as well as for the un-derivatised natural products: a signal–noise ratio higher than 3 for the H-3 doublet was obtained for all compounds with less than 128 transients. Chemical shifts recorded under LC-NMR conditions differed slightly from those recorded in deuterated chloroform. Comparison of LC-^{1}H-NMR spectra of (*R*)-MTPA **1a** and (*S*)-MTPA **1b** derivatives showed significant chemical shift differences for the protons near to the MTPA ester (Fig. 2). These diagnostic shifts were used for the determination of absolute configuration. Suppression of the acetonitrile solvent resonance (δ 2.00) hampered a clear assignment of the signals between δ 1.60 and 2.20. For the unambiguous chemical shift attribution of each proton for both Mosher's esters **1a** and **1b**, ^{1}H-^{1}H WET COSY spectra were recorded (Fig. 3). With these two-dimensional correlation experiments, precise chemical shift assignments for H-1′, H-3′ and H-4′ were possible. Differences of all chemical shifts of (*S*)-MTPA **1a** and of (*R*)-MTPA **1b** were calculated using equation (1). Positive δH values were recorded for the α-pyrone moiety (C-1′), which was located on the right side of the Mosher model: negative

δH values for C-3′/C-5′ indicated that the alkyl chain had to be located on the left side of the model (Fig. 4). Finally, the proton geminal to the esterified hydroxyl group was located on the opposite side of the plane and the absolute configuration at C-2′ was determined using the priority rule of Cahn–Ingold–Prelog. This arrangement indicated a sinistrorse rotation and confirmed the *S* absolute configuration for the asymmetric centre C-2′ (Fig. 4).

The same type of LC-NMR analyses were performed on the α-pyrone **2** and its Mosher's ester derivatives **2a** and **2b**. As in the case of **1**, useful differences in the proton chemical shifts owing to the diamagnetic effect of the benzene ring of the MTPA moiety were observed. Assignment of the ^{1}H signals between δ 1.20 and 2.20 were obtained via a ^{1}H-^{1}H WET COSY experiment. Calculation of the $\Delta\delta_H$, together with the model proposed by Mosher, permitted assignment of the *S, S* configuration for both asymmetric centres at C-4′ and C-6′ (Fig. 5).

All results presented above were obtained with 250 μg of purified MTPA derivatives. In order to minimise the consumption of starting material and to simplify sample clean-up, direct LC-NMR analysis of the whole reaction mixture for the determination of absolute configuration was attempted. Two aliquots (50 μg) of α-pyrone **1** were esterified, one with (*R*)-MTPA and the other with (*S*)-MTPA, following the procedure described in the experimental part (micro-reaction esterification). At the end of the reaction, the excess of dichloromethane was removed under a gentle nitrogen flux. Aliquots (10 μL) equivalent to 5% of the residual reaction mixture were analysed by LC-ES/MS using the same isocratic conditions as described for LC-NMR in order to verify the completion of the esterification reaction in both cases. A molecular ion $[M+H]^+$ at *m/z* 491 (α-pyrone 274 Da + MTPA 216 Da) was observed for the main peak, confirming the esterification of **1** by MTPA chloride. The remaining reaction mixture was then submitted to stop-flow LC-NMR analysis. Pyridine, excess of MTPA and traces of starting material eluted rapidly, while the ester derivatives eluted between 8 and 12 min. Under these conditions, good quality spectra were recorded (signal–noise ratio > than 4 for H-3; NT = 1024) as shown in Fig. 6. Similar results were obtained to those measured using a sample size of 250 μg, thus demonstrating the usefulness of the method when working with restricted amounts of starting material.

The present study has demonstrated that the chemical shifts of the protons of Mosher's ester derivatives established by stop-flow LC-^{1}H-NMR are comparable with those obtained by conventional ^{1}H-NMR analysis. Analysis of $\Delta\delta_H$ gave the correct absolute configuration of both α-pyrones **1** and **2**. Micro-reaction combined with LC-NMR analysis seems to be very promising for a rapid and efficient determination of the absolute configuration of natural products which can only be isolated in very small amounts. The method can be applied to mixtures of compounds which are difficult to isolate on the preparative scale since separation of the Mosher's esters is performed prior to NMR detection.

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Detection of aristolochic acid in Chinese phytomedicines and dietary supplements used as slimming regimens

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Abstract

Over the last 10 years, numerous cases of intoxications, leading for the most part to end-stage renal failure, have been reported after consumption of slimming regimens made of Chinese herbal preparations. These intoxications were associated with species of the *Aristolochia* genus, such as *Aristolochia fangchi* (Aristolochiaceae), known to contain very nephrotoxic and carcinogenic metabolites named aristolochic acids. Several commercial dietary supplements, teas and phytomedicines used as slimming regimens were analysed for their aristolochic acid I content. A preliminary detection of this toxic compound was made by thin-layer chromatography. The presence of aristolochic acid I in these preparations was confirmed by a HPLC/UV-DAD/MS analysis. A quantitative determination of aristolochic acid I was also achieved in the incriminated preparations using both UV and MS detection. Out of 42 analysed preparations, four were found to contain aristolochic acid I and two were suspected to contain aristolochic acid derivatives. Immediate removal of these products from the Swiss market was called for.

Keywords: Aristolochic acid; Intoxication; LC/UV; LC/MS; Aristolochiaceae; *Aristolochia fangchi*

1. Introduction

Several cases of end-stage renal failure after consumption of slimming regimens involving Chinese plant preparations have recently been described in the literature (Vanherweghem et al., 1993; Pena et al., 1996; Tanaka et al., 1997; Stengel and Jones, 1998; Vanherweghem, 1998; Chang et al., 2001; Yang et al., 2000). These intoxications were due to an accidental contamination of these preparations by species belonging to the *Aristolochia* genus (Vanhaelen et al., 1994). The confusion was attributed to similar Chinese vernacular denominations between plants contained in these phytomedicines and some species belonging to the *Aristolochia* genus, such as *Aristolochia fangchi* Y.C. Wu (Flurer et al., 2000). The most common confusion occurs with *Stephania tetrandra* S. Moore (Capparaceae) (Chinese name: fangchi or hanfangji) that is replaced by the very toxic *Aristolochia fangchi* (Chinese name: guangfangchi). The *Aristolochia* genus contains more than 800 herbaceous or shrubby, often climbing, species growing in both temperate and tropical regions and is well known to contain very nephrotoxic and carcinogenic compounds named aristolochic acids. These nitrophenanthrene derivatives were indeed associated with severe renal insufficiency and urothelial carcinoma after their DNA adducts were found in related human tissue samples (Nortier et al., 2000; Stiborova et al., 2000). The mutagenic properties of several metabolites of aristolochic acids I and II formed by rat liver were also determined experimentally (Schmieser et al., 1986). The presence of aristolochic acids I and II has already been reported in Asian medicinal plants as well as in slimming products sold on the Asian market (Zhu and Phillipson, 1996; Hashimoto et al., 1999; Lee et al., 2002).

Abbreviations: APCI, atmospheric pressure chemical ionisation; SPE, solid phase extraction; TLC, thin-layer chromatography.

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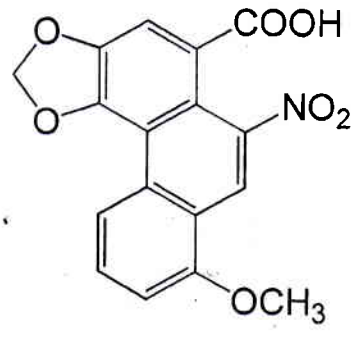
For these reasons, several health institutions, including the US Food and Drug Administration (FDA), have recently published safety information related to the presence of aristolochic acids in plant preparations in order to prevent further cases of intoxication (information available at web address: http://www.cfsan.fda.gov/~dms/ds-bot.html). Several analytical methods using layer chromatography (Pharmacopoeia of the People's Republic of China, 1988; Vanherweghem et al., 1993), fluorometric assay (Rao et al., 1975), gas–liquid chromatography analysis equipped with a flame-ionisation detection (Rao et al., 1975), HPLC coupled to a UV spectrophotometric detector (HPLC/UV) (Nishida and Fukami, 1989; Tsai et al., 1993; Hashimoto et al., 1999; Lee et al., 2002) or to a quadrupole ion-trap mass spectrometer (HPLC/MS/MS) (Kite et al., 2002) have already been employed for the detection and quantification of aristolochic acids. With the same aim, we recently developed two different methods—a thin-layer chromatographic (TLC) assay and a LC/UV-DAD/MS analysis—for a rapid, specific, sensitive and quantitative determination of aristolochic acid I, the major toxic derivative found in *Aristolochia fangchi*, in complex herb mixtures (Fig. 1). A strategy combining these two methods was applied to detect the presence of aristolochic acid I in Chinese phytomedicines commercialised in Switzerland.

2. Materials and methods

2.1. Origin of the analysed samples

The analysed samples were obtained from private or industrial sources after advertisement was made in professional journals and local newspapers. All the selected samples were declared as Chinese plant mixtures used as slimming regimens in the treatment of obesity. Samples available in amounts too small for analysis were either obtained in larger quantities from the same source or withdrawn from the study. These phytomedicines were either ground vegetable material or plant extracts in the format of tea bags, pills, powders, teas, capsules, pellets, granules or tablets.



Aristolochic acid I

Fig. 1. Structure of aristolochic acid I.

2.2. Standard compounds and reagents

Aristolochic acid I used as a reference compound for the analysis was purified by preparative TLC from a mixture of aristolochic acids purchased from Sigma (reference no. A-5512) containing 67% of aristolochic acid I. This mixture was first eluted on a silicagel 60 F_{254} TLC glass-backed plate (Merck) with $CHCl_3$:MeOH: acetic acid (65:20:5, by vol.) before being submitted to a second migration on a RP-18 WF_{254} TLC glass-backed plate (Merck) using MeOH:H_2O (35:65). The purity of aristolochic acid I was checked by LC/UV-DAD under the conditions described in the LC/MS section. Diphenylamine (purum) was purchased from Fluka (reference no. 42761).

2.3. Preparation of the samples for TLC and HPLC analysis

An amount of 5 g of plant material (pills, capsules, plant powder or plant extract) was extracted with 100 ml of boiling MeOH for 3 h. After filtration, the sample was evaporated to dryness under reduced pressure and freeze-dried. A 20-mg/ml methanolic solution of this extract was used for the qualitative detection of aristolochic acid I by TLC and HPLC/UV-DAD/MS analysis. To perform quantitative measurements, an amount of 50 mg of this extract was dissolved in 1 ml of solvent (10% MeOH and 90% H_2O) and eluted with 5 ml of H_2O by solid phase extraction (SPE) using a 3-ml Chromabond® C18 Hydra column (Macherey-Nagel) previously stabilised with 10 ml of H_2O. A quantitative elution of aristolochic acid I was ensured by 20 ml of a MeOH:THF solution (80:20, v/v). This percolate was then evaporated to dryness under reduced pressure and freeze-dried before being dissolved in 1 ml of MeOH. Samples prepared for the purpose of quantification were performed in triplicate and precisely weighed.

2.4. TLC conditions

The sample was applied to a silicagel 60 F_{254} TLC glass-backed plate and eluted with $CHCl_3$:MeOH:acetic acid (65:20:2, by vol.). After evaporation of the solvents, the plate was sprayed with a solution of 0.5% diphenylamine in H_2SO_4 60% and heated for 10 min at 100 °C (blow-dryer or oven). Under these conditions, aristolochic acid I was detected under visible light as a dark blue or black spot which developed a yellow fluorescence when observed at 366 nm. Under these conditions

of separation, an approximate HR_f of 72 was calculated for aristolochic acid I.

2.5. HPLC/UV-DAD conditions

Reversed-phase HPLC was carried out using an HPLC system HP1100 (Hewlett-Packard, Palo Alto, CA, USA) equipped with a binary pump. A Hewlett-Packard (Walbronn, Germany) 1100 series on-line photodiode array detector (DAD) was used for detection. This instrumentation was controlled by HP Chemstation software. The separation was performed on a C-18 Symmetry® column (250×4.6 mm i.d., 4 μm particle size, Waters, Bedford, MA, USA) with a linear MeOH (0.5% acetic acid):H_2O (0.5% acetic acid) gradient (60:40 to 100:0) in 21 min. The column temperature was set at 30 °C with a flow of 1 ml/min. The DAD-UV detector was set at 254 and 224 nm. A reference mixture of aristolochic acids, mostly composed of aristolochic acids I and II, was employed to optimise the LC separation method. Addition of acetic acid was required to avoid tailing of the compounds of interest during the LC separation. Under these conditions of separation and during the whole period of analysis (several weeks), aristolochic acid I was eluted with a constant retention time between 13.9 and 14.0 min. A volume of 20 μl was injected. Quantification using UV detection was performed at 254 nm. Samples for quantitative analysis were injected in triplicate. Each solution was diluted with MeOH in order to obtain a concentration within the range of the calibration curve.

2.6. MS conditions

MS analyses were performed by atmospheric pressure chemical ionization (APCI) on a Finnigan LCQ ion trap mass spectrometer (FinniganMAT, San Jose, CA, USA). The APCI optimised parameters were: capillary temperature 150 °C, vaporiser temperature 450 °C, corona needle current 5 μA and sheath gas flow 60%. These parameters were optimised by Flow Injection Analysis (FIA) using aristolochic acid I as reference compound. Spectra (150–700 amu) were recorded in the positive ion mode. Quantification using MS detection was performed in the full scan mode set between 150 and 700 amu. Samples for quantitative analysis were injected in triplicate.

3. Results

The samples for analysis were first screened in a TLC assay using diphenylamine as detection reagent. Under these conditions, aristolochic acid I was seen as a dark-blue coloured spot under visible light (see Plate 1). A yellow fluorescence was also observed at 366 nm after

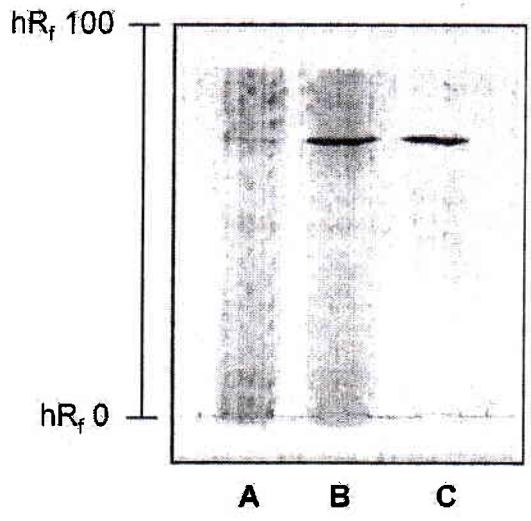


Plate 1. Detection of aristolochic acid I by TLC under visible light in sample 16 (declared as Han- fang ji, *Stephaniae tetrandrae* radix). (A) MeOH extract of sample 16 (20 mg/ml, 10 μl). (B) 1:1 mixture of A and C. (C) Aristolochic acid I (1 mg/ml, 10 μl).

reaction with diphenylamine. This method allowed the detection of quantities of aristolochic acid I as small as 1 μg in visible light and 0.2 μg at 366 nm. Aristolochic acid I was easily detected by TLC in samples 16 and 17 and was suspected in sample 22.

A LC/DAD-UV/MS analysis was then employed to assess the presence of aristolochic acid I in the studied plant preparations and if necessary, to provide quantification. Results of the different analysed samples are presented in Table 1. Aristolochic acid was detected through the presence of a chromatographic peak eluting at about 13.8 min exhibiting a quite typical UV spectrum with maxima at 224, 251 (sh), 321 and 393 nm as well as characteristic ionised fragments at m/z 295 $(M\text{-}NO_2)^+$ m/z and 324 $(M\text{-}OH)^+$ in the APCI-MS positive mode (see Table 2). Under the experimental conditions, a detection limit of 2 ng of aristolochic acid I was determined for both UV and SIM-MS measurements. A detection limit of 15 ng was obtained in the full scan MS mode. The presence of aristolochic acid I was unambiguously found in four samples, three of them being declared as Fang ji or Han fang ji preparations (see Table 1). Only traces of the toxic product could be determined in sample 15, while larger amounts were discovered in the three other samples. Aristolochic acid I could not be detected in sample 15 by TLC. The result of the TLC screening for aristolochic acid I was tenuous in the case of sample 22, but the presence of aristolochic acid I could be confirmed through the LC/UV-DAD/MS analysis (Fig. 2). Samples 10 and 21 were free of aristolochic acid I but the presence of other aristolochic acids was strongly suspected, since some compounds in these preparations exhibited UV spectra similar to that of aristolochic acid I, associated with MS ions at m/z 295,

Table 1
Commercial samples analysed for their aristolochic acid I content

No.	Declaration of content	Product presentation	Aristolochic acid I	Remarks
1	Pu-Ehr tea	Tea bags	–	
2	Pu-Ehr tea	Capsules	–	
3	Pu-Ehr tea	Tea bags	–	
4	Black tea	Tea bags	–	
5	Pu-Ehr tea	Capsules	–	
6	–[a]	Granules	–	
8	Green tea, Java tea, anise, blackcurrant	Tea bags	–	
9	Black tea	Tea bags	–	
9	–	Powder	–	
10	*Asari herba*	Powder	–	Suspicion of the presence of aristolochic acid derivatives
11	Pu-Ehr tea	Tea herb	–	
12	Pu-Ehr tea	Capsules	–	
13	Pu-Ehr tea	Pills	–	
14	[b]	Tea herb	–	
15	Fang ji huang qi tang *Stephania* and *Astragalus*	Powder	+	Traces of aristolochic acid I (not visible on TLC)
16	Han fang ji *Stephaniae tetrandrae* radix	Powder	+	Quantification of aristolochic acid I UV: 0.044% MS: 0.040%
17	Ba zheng san (eight herbs)	Powder	+	Quantification of aristolochic acid I UV: 0.009% MS: 0.014%
18	Shu jing huo xue tang	Powder	–	
19	Xin yi san Magnoliae fl.	Powder	–	
20	Chuan mu tong *Clematidis caulis*	Powder	–	
21	Xi xin *Asari herba*	Powder	–	Suspicion of the presence of aristolochic acid derivatives
22	Han fang ji *Sinomenium acutum*	Powder	+	Dubious result on TLC
23	San bi tang	Powder	–	
24	Du huo ji sheng tang *Angelica pubescens*	Powder	–	
25	Long dan xie gan tang *Gentiana longdancao*	Powder	–	
26	Pu-Ehr tea	Capsules	–	
27	–	Pills	–	
28	–	Powder	–	
29	–	Tea herb	–	
30	Green tea	Tea herb	–	
31	–	Tea bags	–	
32	Ginseng tea	Tea bags	–	
33	–	Pellets	–	
34	Shu xin xiao zhi wan	Pellets	–	
35	Jian pi he wei wan	Pellets	–	
36	Yi shen yang yin wan	Pellets	–	
37	Yi qi yang xue wan	pills	–	
38	Xiao feng san	Powder	–	
39	*Ligusticum radix*	Powder	–	
40	Fang ji huang qi tang *Stephania* and *Astragalus*	Powder	–	
41	Black tea	Tablets	–	
42	Xiao feng san	Powder	–	

For reasons of public health care, translated Chinese names of the analysed preparations are given where possible.
[a] – Products obtained without a declaration.
[b] Product declaration only available in Chinese.

324, 342, 354 and 358 expected for aristolochic acid derivatives (Fig. 3 and Table 2). Quantification of aristolochic acid I in samples 16 and 17 was performed using UV and MS detection. These samples were determined as the commercial products containing the highest amounts of aristolochic acid I. Very similar results were obtained using these two different techniques (see Table 1). Sample 16, declared as a single plant preparation, was found to contain about four times more aristolochic acid I than 17, said to be a mixture of eight drugs.

4. Discussion

Owing to its high toxicity, the development of a selective and sensitive method for the detection of aristolochic acid I in complex mixtures is more important than its accurate quantification. The presence of aristolochic acid I and of its derivatives is indeed not acceptable in plant preparations since even traces of these compounds constitute a potential health risk. However, the determination of new toxicological data in the future should be considered as a sufficient motivation to achieve both detection and quantification of aristolochic acid I. The detection of aristolochic acid I in herbal preparations was ensured by a combination of two different techniques, namely a TLC analysis followed by detection of aristolochic acid I through chemical derivatisation and an HPLC separation coupled to UV diode array and mass spectrometry detection. Details concerning the optimisation and the validation of these methods have already been discussed elsewhere (Ioset et al., 2002). The combination of these two techniques proved to be a very efficient strategy in the search for aristolochic acid I in plant preparations. The two samples containing the highest concentrations of aristolochic acid I—samples 16 and 17—could easily be detected by the TLC screening. This method allowed the detection of quantities of aristolochic acid I as small as 1 μg in visible light and 0.2 μg at 366 nm, through the presence of its strongly oxidative NO_2 group. This reaction can be considered as very specific in the context of plant chemistry since only very few natural products have been characterised to contain this type of functional group (see Fig. 2). Two additional samples—samples 15 and 22—were also found to contain aristolochic acid I after LC/UV-DAD/MS analysis. The advantages of this technique are the specificity and sensitivity of both UV and MS detection. Indeed, aristolochic acid I exhibited a typical UV spectrum as well as a strongly ionised characteristic fragment at m/z 295 $(M\text{-}NO_2)^+$ in the APCI-MS mode due to the loss of the labile NO_2 functional group. The sensitivity of the detection was in the ng range for both UV and MS detection. These results cannot of course guarantee the total safety of use of the analysed preparations since it is not yet known whether undetectable traces of aristolochic acids can still have toxic effects if taken regularly over a long period. Other parameters such as consumer sensitivity to aristolochic acid, quantities of ingested preparations, predisposition to renal insufficiency, should also be considered in the risk evaluation of intoxication. Similar limits of detection were obtained for UV (Hashimoto et al., 1999; Lee et al., 2002) and MS (Kite et al., 2002) analysis of aristolochic acid I but the method developed here has the advantage of coupling both kinds of detection in association with a prior rapid concentration of the sample by SPE. A search strategy for aristolochic acid I in herbal preparations was also proposed using a TLC pre-screening followed by a LC/UV-DAD/MS confirmative analysis. Both UV and MS detection allowed the quantification of aristolochic acid I in the samples undergoing analysis, very similar amounts of this toxic compound being determined by these two techniques. The presence of lower amounts of aristolochic acid I in sample 16 compared with those determined for *Aristolochia fangchi* in the literature (Hashimoto et al., 1999) could be explained by the fact that *Aristolochia fangchi*—probably mistaken for the declared *Stephania tetrandra*—had either a lower content of aristolochic acid I or was mixed with other herbs. Sample 17—found to contain only one-quarter of the amount of aristolochic acid I calculated for 16—is probably a mixture of several herbs, as declared by the supplier.

Mass spectrometry was not only useful in the qualitative and quantitative determination of aristolochic acid I, but also suggested the presence of other aristolochic acids in samples 10 and 21 due to characteristic UV spectra associated with MS ions at m/z 295, 324, 342, 354 and 358, specific for aristolochic acid derivatives (see Fig. 3 and Table 2). Such results are not surprising since these two preparations were declared as *Asari herba*, a genus belonging to the Aristolochiaceae family and well known to contain aristolochic acid derivatives (Hashimoto et al., 1999).

Finally, four out the 42 analysed preparations were found to contain aristolochic acid I and the presence of aristolochic acid derivatives was suspected in two other herbal mixtures. Five out of the six incriminated products were provided by the same Chinese herb importer. Immediate removal of the products from the Swiss market was called for. Because of an increased interest for natural alternative medicine, consumers have to face a huge variety of new drug treatments and herbal health supplements to combat overweight. For several reasons, including confusion of close vernacular Chinese names, lack of quality control, misuse in the drug preparation, dosage or indication and also toxicity related to some Chinese traditional herbs, the use of such preparations is not without risk. The results of this work show the importance for a better survey of herbal

Table 2
Some important aristolochic acids and their expected ions

Aristolochic acids	Molecular weight	Expected ions*
I, III	341	342, 324, 295
Ia, IIIa	327	328, 310, 281
II	311	312, 294, 265
IV, V, VI, VII	371	372, 354, 325
IVa, Va, VIa, VIIa	357	358, 341, 311

* Through protonation $[M+H]^+$, loss of OH $[M\text{-}17]^+$ and NO_2 $[M\text{-}46]^+$ groups.

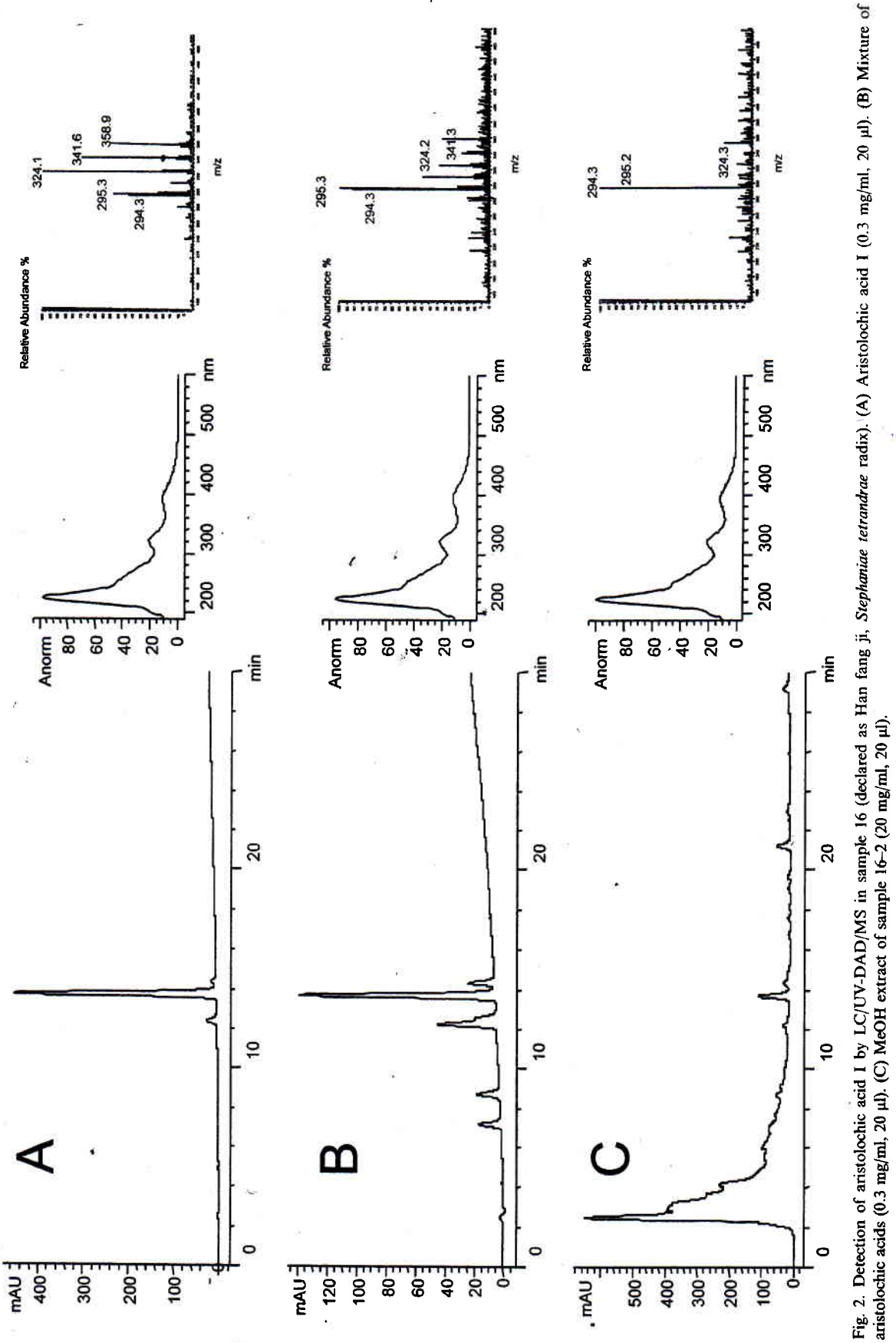


Fig. 2. Detection of aristolochic acid I by LC/UV-DAD/MS in sample 16 (declared as Han fang ji, *Stephaniae tetrandrae* radix). (A) Aristolochic acid I (0.3 mg/ml, 20 μl). (B) Mixture of aristolochic acids (0.3 mg/ml, 20 μl). (C) MeOH extract of sample 16-2 (20 mg/ml, 20 μl).

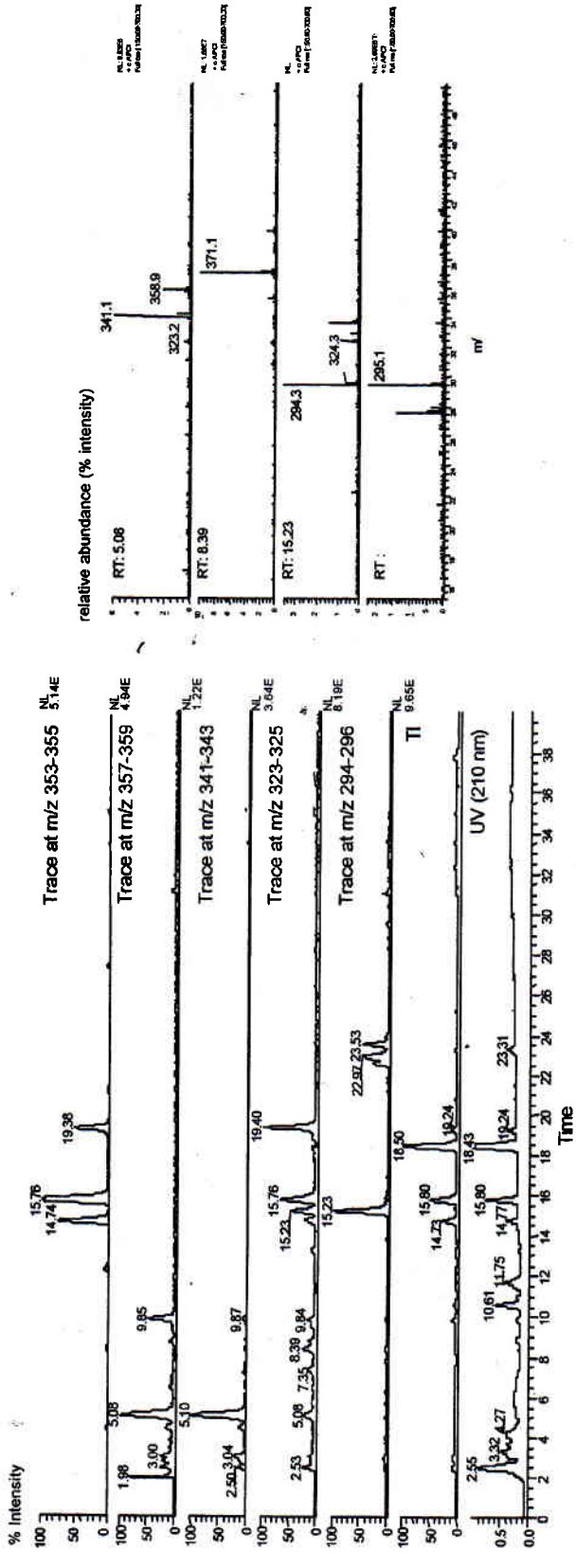


Fig. 3. LC/UV-DAD/MS analysis of sample 10 declared as *Asari herba*. Left-hand side: mass traces corresponding to ions specific for aristolochic acids. Right-hand side: mass spectra of four selected compounds showing ions specific for aristolochic acids.

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M.H. Rafamantanana, E. Rozet, **G.E. Raoelison**, K. Cheuk, S. Urverg Ratsimamanga, Ph. Hubert, J. Quetin-Leclercq. An improved HPLC-UV method for the simultaneous quantification of triterpenic glycosides and aglycones in leaves of *Centella asiatica* (L.) Urb (APIACEAE). *Journal of Chromatography B* 2009, **877**, 2396-2402.