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Determination of kava lactones in food supplements by liquid chromatography–atmospheric pressure chemical ionisation tandem mass spectrometry

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Abstract

Reversed-phase liquid chromatography and detection with atmospheric pressure chemical ionisation tandem mass spectrometry was used for the determination of kava extracts in herbal mixtures. One percent of kava extract can be detected, corresponding to approximately 0.05–0.2 mg/g of the individual kava lactones kavain, dihydrokavain, yangonin, desmethoxyyangonin, methysticin and dihydromethysticin. Reliable quantification is obtained from concentrations of 0.25–1 mg/g, depending on the compound. At these concentration levels, the relative standard deviations were 10–14%. Validation showed good linearity and recoveries for all the kava lactones with the exception of yangonin. During method development, degradation of yangonin was observed. The degradation product was identified by nuclear magnetic resonance (NMR) as *cis*-yangonin. The method was applied to the analysis of commercial herbal products available in the Dutch market before and after market restrictions of kava-containing preparations. The results showed that even though 'old' products contained kava extract, the new formulations were negative on kava lactones. *cis*-Yangonin was also present in the herbal products.

Keywords: *Piper methysticum*; Liquid chromatography; Tandem mass spectrometry; Kava lactones

1. Introduction

During the last decades, the use of kava in various herbal preparations has increased in Europe and in the U.S. [1,2]. Kava originates from the South Pacific where it is still prepared as a beverage and consumed for its intoxicating effects [3]. The effects described in the literature include anxiolytic effect, sedation, hypnosis, analgesia and muscle relaxation. Chronic use of kava has also been associated with side effects such as allergic skin reaction, reversible gastrointestinal disturbance and several cases of severe hepatotoxicity [4,5]. Even though the evidence is not fully convincing, regulatory agencies in Canada, U.S. and several European countries have taken precautions and warned consumers or removed kava-containing products from the food supplement market [4–6]. Following other European countries such as Germany, in summer 2003, the authorities in The Netherlands have banned the use of kava extract in food supplements and restricted it to medicinal use. This situation created a need for robust and selective analytical methods which can be used (i) for quantification of kava components at relatively high concentrations in quality control of pure kava extracts and phytotherapeutics and (ii) for detection of kava extracts at low concentration levels in mixtures of various herbal extracts sold as food supplements with claims of lowering anxiety.

So far, 18 compounds have been identified in the kava root extracts [7,8]. The compounds to which most of the activity of kava is assigned are the kava lactones desmethoxyyangonin, dihydrokavain, yangonin, kavain, dihydromethysticin and methysticin (Fig. 1).

For the qualitative and/or quantitative determination of these compounds, several methods have been described in the literature varying from gas chromatographic (GC) [1,6,9,10] and liquid chromatographic (LC) [11,12] to nuclear magnetic resonance (NMR) [7]. The lactones are aromatic and not very polar, which makes them amenable to both GC and LC analysis. Most quantitative methods use LC in combination

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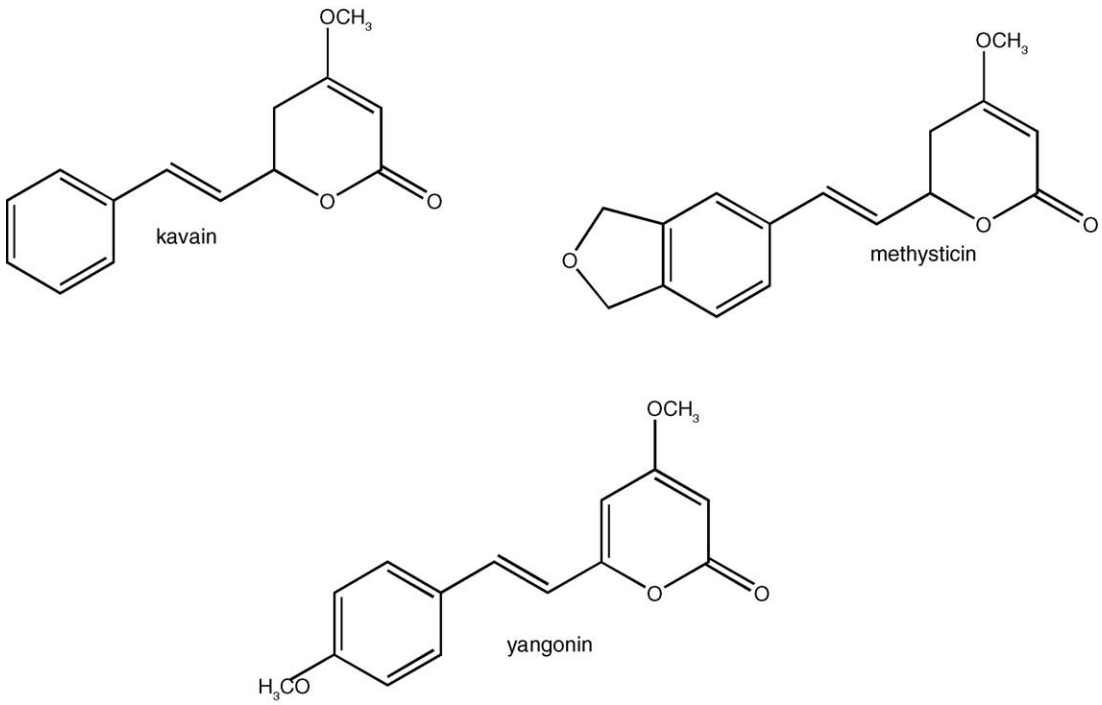


Fig. 1. Structures of the major kava lactones kavain (styrene bond is saturated in dihydrokavain), methysticin (styrene bond is saturated in dihydromethysticin) and yangonin (desmethoxyyangonin does not contain a methoxy functionality on the benzene ring).

with ultraviolet (UV) detection. Drawback of UV detection is the lack of selectivity. Kava lactones need to be baseline-separated even in pure kava extracts. UV detection and quantification in herbal mixtures is even more complex and time consuming, leading to long analysis times. GC with mass spectrometric (MS) detection has been used for identification purposes and for residue determination of kava lactones in complex matrices such as human hair [6]. The method is selective and sensitive, but involves concentration (evaporation) steps. Detection by electrospray ionisation (ESI)-MS has also been described in combination with reversed-phase LC and applied for qualitative analysis. By combining single MS and UV, 10 known kava lactones were detected in powdered kava root extract and 3 structurally related compounds were detected but not identified [11]. The authors observed degradation of yangonin, but were not able to identify the degradation product by LC–UV–MS. NMR is suitable for identification but was not used in this specific case. We were not able to find literature references where the use of atmospheric pressure chemical ionisation (APCI) is used for ionisation. The lactones are moderately polar; therefore, APCI in combination with MS/MS should result in good sensitivity, selectivity and generally in a broader linear range than ESI.

The main goal of this study was to develop and validate a fast and selective method for the determination of the six major kava lactones in kava extracts and herbal mixtures containing kava extract. For quality control of products with restricted use of kava, the method should detect the presence of 1% of kava extract in a mixture. Ten percent of kava extract is usually added to mixtures for sufficient effect. At this level, the method should give reliable quantitative results. The secondary aim of this study was to perform a limited investigation on the herbal preparations available in the Dutch market and their compliance to the new restrictions of kava use.

2. Experimental

2.1. Extracts and food supplements

Kava extract and St. John's Wort extract in capsules were purchased from local drugstores. The total kava lactone content in the kava extract was indicated as 52–57 mg/capsule. The weight of the extract was 600 mg/capsule.

A total of 10 herbal food supplements with claims such as "soothes anxiety" were purchased at local drugstores. The dates of purchase were 2001–2003; all food supplements were within the expiry date. Three 'old' products were purchased before the market restrictions in The Netherlands. According to the ingredient declaration, these products contained kava extracts. Seven products were purchased about 3 months after the restrictions and include two new formulations of 'old' products. Kava was not listed as one of the ingredients.

2.2. Reagents and standards

A kava kit containing the six major kava lactones (article number 11310-10K) was purchased from Chromadex (Santa Ana, CA, USA).

Formic acid, acetonitrile and ammonium acetate were from Merck (Darmstadt, Germany), methanol from Biosolve (Valkenswaard, The Netherlands).

2.3. Validation samples

Due to limited availability of pure standards and non-availability of reference materials, the validation of the proposed method was performed using a commercial kava extract characterised by the proposed method (see also Section 3). The calculated concentrations of the individual kava lactones were 2.4% of kavain, 2.1% of dihydrokavain, 1.3% of dihydromethysticin, 0.93% of methysticin, 2.3% of yangonin and 0.6% of desmethoxyyangonin. A blank matrix, St. John's Wort, was spiked with 1, 5 and 10% of the kava extract. For the sample spiked with 1% of kava extract, this results in individual concentrations of 0.24 mg/g of kavain, 0.21 mg/g of dihydrokavain, 0.09 mg/g of methysticin, 0.13 mg/g of dihydromethysticin, 0.23 mg/g of yangonin and 0.06 mg/g of desmethoxyyangonin.

2.4. Sample preparation

Two hundred milligrams of sample are carefully weighed and placed into a plastic 50 ml centrifuge tube. Fifty millilitres of HPLC-grade methanol are added using a dispenser. The extraction mixture is placed in an ultrasound bath for 30 min. The samples are centrifuged for 15 min at 3000 × *g*. One millilitre of the supernatant is then diluted with 1 ml of methanol and 2 ml of Milli-Q water. An aliquot is transferred to an autosampler vial and is ready for injection. The extracted samples are analysed within 48 h after extraction.

2.5. LC–MS/MS

The analyses are performed on an Alliance Waters 2690 from Waters (Etten-Leur, The Netherlands) LC system equipped with an autosampler and coupled to a Finnigan LCQ Classic ion trap mass spectrometer from ThermoFinnigan (Hampstead, UK). The autosampler trays are maintained at 8 °C. Ten microlitres of the methanol extract is injected onto the Waters Xterra RP C_{18} 3.5 μm × 3 mm × 150 mm column. The compounds are separated using a gradient of water (A) and acetonitrile (B); 0.1% formic acid and 0.1% ammonium acetate is added to both solvents A and B. The gradient with a flow of 0.3 ml/min is started at 40% B and linearly increased to 99% B in 20 min. The original composition of 40% B is restored linearly within 1 min and the column is conditioned with this solvent composition for another 4 min before injecting the next sample. In combination with the used flow, this is a total of 3.5 column volumes; this time suffices to restore the original composition of the mobile phase. The total analysis time is 25 min. The column temperature is maintained at 40 °C. The eluting compounds are ionised using the standard Finnigan APCI interface in the positive mode. The temperature of the capillary is 150 °C, the sheet gas flow is 27 (arbitrary units, approximately 0.4 l/min) and the auxiliary gas is 3 (arbitrary units, approximately 1 l/min). The discharge current is 5 mA, the capillary voltage is 34 V. The tube lens offset is 55 V.

For each compound, one transition is monitored; for methysticin, three transitions are selected. The MS conditions were optimized in order to maximize the selected signals. For an overview of the ions, retention times and collision energies, see Table 1.

2.6. Calibration

Pure compounds from the kava kit are dissolved in methanol and diluted with water–methanol (1:1). Stock solutions with concentrations of approximately 0.5 mg/ml were prepared. Calibration standards at five levels are prepared in the range from 0.2 to 5 mg/l. In this range, linearity was determined (see Section 3). Due to the price and limited availability of the pure standards, for routine analysis, calibration is performed only at two concentration levels, 0.2 and 5 mg/l, at the beginning and end of each series.

2.7. NMR

Prior to the NMR analysis, 2 mg of yangonin were dissolved in 0.8 ml of methanol-d_4. The fresh sample and a sample stored for 36 h at room temperature were analysed.

1D ^{1}H NMR spectra were recorded on a Bruker (Bremen, Germany) AVANCE 600 MHz spectrometer using a proton NMR set-up operating at a temperature of 300 K. Free induction decays were collected as 32K data points with spectral width of 10.776 Hz; 30° pulses were used with an acquisition time of 3.04 s and a relaxation delay of 5 s. The spectra were acquired by accumulation of 256 FIDs. The spectra were processed using the standard Bruker software. An exponential window function with a line broadening of 0.3 Hz and a manual baseline correction was applied. Chemical shifts were referenced to the methanol signal ($\delta = 3.300$).

3. Results and discussion

3.1. LC–MS

Rather than developing a specific method for the determination of only these particular compounds, we preferred to test existing extraction and separation methods. Analysis of kava lactones in herbal preparations can then be combined with the analysis of other bioactive compounds often present in these types of samples.

For separation, acidic methanol–water and acidic acetonitrile–water gradients were tested. Baseline separation for all compounds was not achieved in combination with a 'reasonable' analysis time, i.e. less than 35 min. An acidic acetonitrile–water gradient was chosen which results in a run time of 25 min including the conditioning of the

Table 1
MS/MS of the investigated kava lactone

Retention time (min), compound, @ collision energy (%)	*m/z* ion (%)	Assignment
7.76, Kavain, @ 34	231[a]	$[M+H]^+$
	213 (50)	$[M+H-H_2O]^+$
	199 (25)	$[M+H-CH_3OH]^+$
	189 (50)	$[M+H-C_2H_2O]^+$; loss of ketene
	185 (*100*)	$[M+H-HCOOH]^+$; *loss of formic acid*
	115 (25)	$[M+H-C_4H_8O_3]^+$; loss of methoxy-dihydrofuranone
7.85, Dihydrokavain, @ 32	233[a]	$[M+H]^+$
	215 (25)	$[M+H-H_2O]^+$
	201 (18)	$[M+H-CH_3OH]^+$
	187 (*100*)	$[M+H-C_2H_2O]^+$; *loss of ketene*
	183 (15)	$[M+H-HCOOH]^+$; loss of formic acid
	117 (12)	$[M+H-C_4H_8O_3]^+$; loss of methoxy-dihydrofuranone
10.30, Desmethoxyyangonin, @ 36	229[a]	$[M+H]^+$
	201 (*100*)	$[M+H-CO]^+$
	169 (10)	$[M+H-H_2O-C_2H_2O]^+$; loss of water and ketene
	131 (15)	$[M+H-C_4H_2O_3]^+$; unknown loss of neutral
10.74, Yangonin, @ 36	259[a]	$[M+H]^+$
	231 (*100*)	$[M+H-CO]^+$
	199 (5)	$[M+H-H_2O-C_2H_2O]^+$; loss of water and ketene
	161 (10)	$[M+H-C_4H_2O_3]^+$; unknown loss of neutral
7.89, Dihydromethysticin, @ 30	277[a]	$[M+H]^+$
	259 (10)	$[M+H-H_2O]^+$
	245 (12)	$[M+H-CH_3OH]^+$
	227 (10)	$[M+H-H_2O-CH_3OH]^+$
	231 (8)	$[M+H-C_2H_2O]^+$; loss of ketene
	161 (*100*)	$[M+H-C_4H_8O_3]^+$; *loss of methoxy-dihydrofuranone*
8.03, Methysticin, @ 30	275[a]	$[M+H]^+$
	257 (*75*)	$[M+H-H_2O]^+$
	243 (*80*)	$[M+H-CH_3OH]^+$
	229 (18)	$[M+H-H_2O-C_2H_2O]^+$
	233 (18)	$[M+H-C_2H_2O]^+$; loss of ketene
	159 (*100*)	$[M+H-C_4H_8O_3]^+$; *loss of methoxy-dihydrofuranone*

Italics: monitored ions (transitions).
[a] Precursor ion.

column. Three compounds, kavain, dihydrokavain and dihydromethysticin (Fig. 2), were not baseline-separated, but the selective detection by LC–MS can distinguish them sufficiently for a reliable identification and quantification. As expected, due to the moderate polarity of the studied compounds, we found APCI in the positive ionisation mode sufficiently sensitive.

For all compounds, the proton adducts, and in four cases also low-intensity ammonium adducts were observed in the full-scan (single MS) mode. Methysticin appeared to be the least stable of the studied compounds; degradation and fragmentation in the source were observed. For all compounds, $[M+H]^+$ was selected for MS/MS. An overview of the main product ions, their intensities and proposed assignment is given in Table 1.

3.2. Extraction

Acetonitrile, methanol, ethanol, dichloromethane (DCM) and ethyl acetate (EtAc) were tested as extraction solvents. Two hundred milligrams of a powdered kava preparation were extracted with 50 ml of each solvent. DCM and EtAc were evaporated to dryness and reconstituted in the mobile phase (10% acetonitrile in water). Duplicate extraction experiments were performed and the extracts were analysed by the proposed method. The results (not shown) were found to be similar for all solvents. Slightly lower recoveries were observed for dihydromethysticin and dihydrokavain when DCM was used. This solvent also showed the highest variance in the duplicates. Methanol is commonly used for extraction of herbal products (e.g. hypericine from St. John's Wort, often used in combination with kava), it is also less toxic than acetonitrile. This solvent was therefore selected for further experiments. After dilution, the methanol extract is injected directly onto the LC–MS system, without any evaporation steps.

3.3. Degradation of yangonin and identification of degradation product

During method development, degradation of yangonin was observed if standards were prepared several days

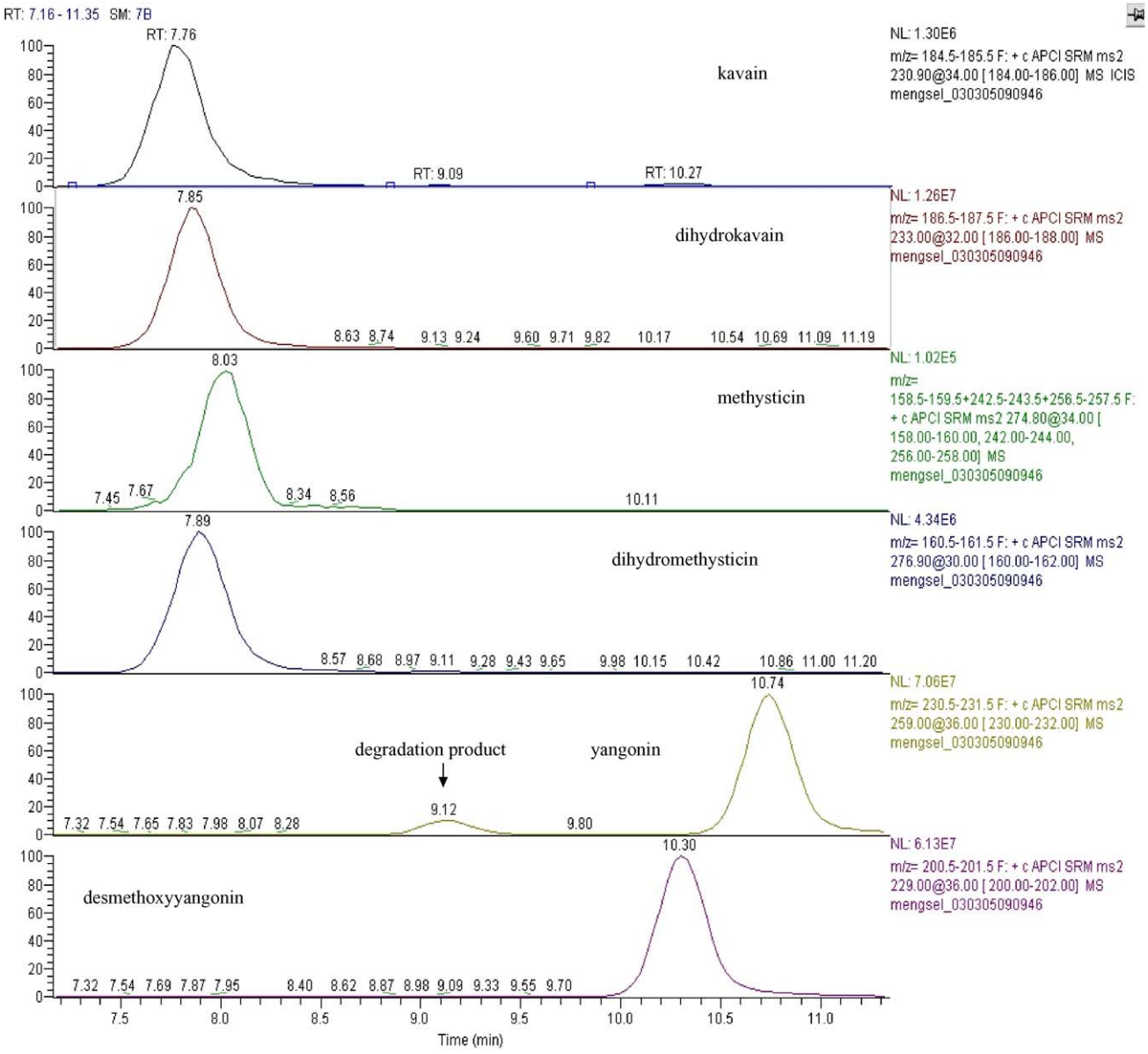


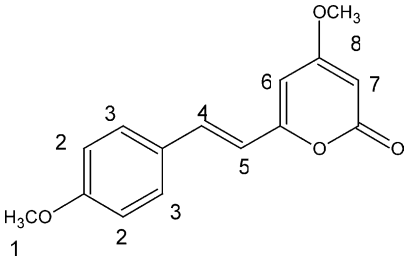
Fig. 2. Selected reaction monitoring (SRM) chromatogram of a standard mixture of kava lactones. For conditions, see Section 2.

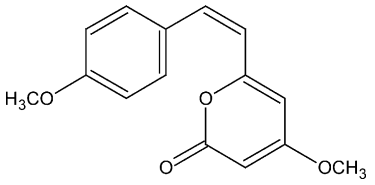
before analysis. This is in agreement with the observation of He et al. [11]. Our experiments also showed that the degradation product has the same molecular weight as yangonin but elutes significantly earlier (Fig. 2). The fragmentation of the $[M+H]^+$ gives an identical product ion spectrum (not shown) as that of yangonin. Based on this information, the degradation product could either be a *cis*-isomer of yangonin or an isomer with the methoxy functionality on the benzene ring in the *meta*-position [1]. 1D ^{1}H NMR spectra of a fresh (A) and a 36-h-old yangonin solution (B) stored at room temperature were acquired. Two sets of chemical shifts are observed in the 'old' yangonin solution confirming the degradation of the compound under these conditions. Chemical shifts and first-order coupling constants, given in Table 2, indicate a *trans–cis* conversion of yangonin in solution (see structure in Table 2).

Formation of *cis*-isomers of natural products by, e.g. thermal treatment was reported before [13]. The significantly lower retention time of the *cis*-isomer can be explained by the better solubility of this compound in water due to the compacter structure. For practical reasons, it was assumed that *cis*-yangonin has the same MS sensitivity as yangonin. During validation and also in further applications of the proposed method, yangonin is reported as the sum of yangonin and *cis*-yangonin.

Table 2
Chemical shifts (ppm) and observed first-order coupling constants (±1 Hz) of yangonin and the degradation product (proposed structure: *cis*-yangonin)

Structure of yangonin/*cis*-yangonin





Compound	H-1a,b	H-2a,b ($J_{2a,3a}$, $J_{2b,3b}$)	H-3 ($J_{2a,3a}$, $J_{2b,3b}$)	H-4 ($J_{4,5}$)	H-5 ($J_{4,5}$)	H-6	H-7	H-8
Fresh solution A—yangonin	3.873	6.940 (9)	7.529 (9)	6.701 (16)	7.387 (16)	6.172	5.582	3.819
Old solution B—yangonin	3.873	6.940 (9)	7.530 (9)	6.701 (16)	7.388 (16)	6.172	5.582	3.819
Old solution B—*cis*-yangonin	3.828	6.887 (9)	7.374 (9)	6.056 (13)	6.801 (13)	6.091	5.559	3.805

3.4. Validation of the method

Due to the non-availability of sufficient amounts of pure standards, the validation was performed using both pure standards and a commercial kava extract sold as food supplement and characterised by the proposed method. The calculated concentrations, 0.24 mg/g of kavain, 0.21 mg/g of dihydrokavain, 0.13 mg/g of dihydromethysticin, 0.09 mg/g of methysticin, 0.23 mg/g of yangonin and 0.06 mg/g of desmethoxyyangonin, were confirmed by standard addition of the pure standards to this extract at approximately the same concentrations (results not shown). In this extract, the degradation product of yangonin was present even if fresh extracts were prepared. The sum of the kava lactones is in good agreement with the specification of the product.

Linearity of the method was determined in the range 0.2–5 mg/l. The method is linear for all compounds, with r^2 ranging from 0.994 (yangonin) to 0.999 (kavain).

Repeatability and recovery were determined by analysing a St. John's Wort extract spiked with 1, 5 and 10% of the commercial powder kava extract. The spiking levels using this particular extract gave concentrations of 0.24, 0.12 and 2.4 mg/g of kavain, but only 0.05, 0.2 and 0.5 mg/g of desmethoxyyangonin (see also Section 2). Each spiked level was analysed in triplicate.

Recovery was calculated by comparing the levels of the individual kava lactones in the kava extract and the theoretical levels in the fortified samples. Each concentration level was analysed in triplicate. The recoveries, shown in Table 3, vary from 40% for (total) yangonin at the 1% fortification level to 118% for dihydrokavain at the 5% fortification level.

The low recoveries of yangonin were not investigated further, but could be caused by the matrix (St. John's Wort). Repeatability was determined as the RSD of the recovery experiments and is also shown in Table 3. For the lowest fortification level, the RSDs vary from 46% for methysticin to 14% for desmethoxyyangonin. For the two higher fortification levels, the RSDs are lower than 14% for all kava lactones.

For the calculation of the limits of detection (LODs) and limits of quantification (LOQs), the two different applications of the proposed method were considered: (i) quantitative determination of the individual kava lactones in herbal preparations and (ii) detection of the presence of kava extract in herbal mixtures. For the latter application, the LOD of the kava extract is more relevant than the LOD of the individual compounds; the LOQ is not relevant. For the former application, the LODs and LOQs of the individual compounds are relevant.

The LODs of the individual compounds were calculated as $3 \times s_0$/intercept, where s_0 is the standard deviation of five different 'blank' herbal extracts analysed in duplicate. The calculated LODs range from 0.001 mg/g (desmethoxyyangonin) to 0.01 mg/g (kavain) of herbal product. Based on the validation results, the LOD for kava extract was set to 1%. Reliable quantification of the individual compounds (LOQ, RSDs < 15%) is possible from 1 mg/g for kavain, dihydrokavain and dihydromethysticin, 0.25 mg/g for methysticin and yangonin and 0.4 mg/g for desmethoxyyangonin. These concentrations correspond to 5% of kava extract present in the herbal mixture (Table 3). Both the LOD level for the kava extract and the LOQ levels of the individual lactones are sufficient for application in quality control of herbal products. If necessary, these levels can be improved by reducing the dilution during extraction (see Section 2).

Table 3
Recovery of individual kava lactones from St. John's Wort extract spiked with kava extract at three concentrations

Compound	Recovery and repeatability (%)		
	Level[a] = 1%	Level[a] = 5%	Level[a] = 10%
Kavain	65 (20)	65 (10)	65 (13)
Dihydrokavain	100 (26)	120 (13)	115 (4)
Methysticin	90 (46)	95 (14)	85 (13)
Dihydromethysticin	75 (20)	75 (12)	85 (13)
Yangonin	40 (20)	45 (11)	45 (5)
Desmethoxyyangonin	80 (14)	85 (13)	90 (11)

The values given in parentheses are RSDs (%).

[a] Spiked with percentage of kava extract. For the concentrations of the individual kava lactones, see Section 2.

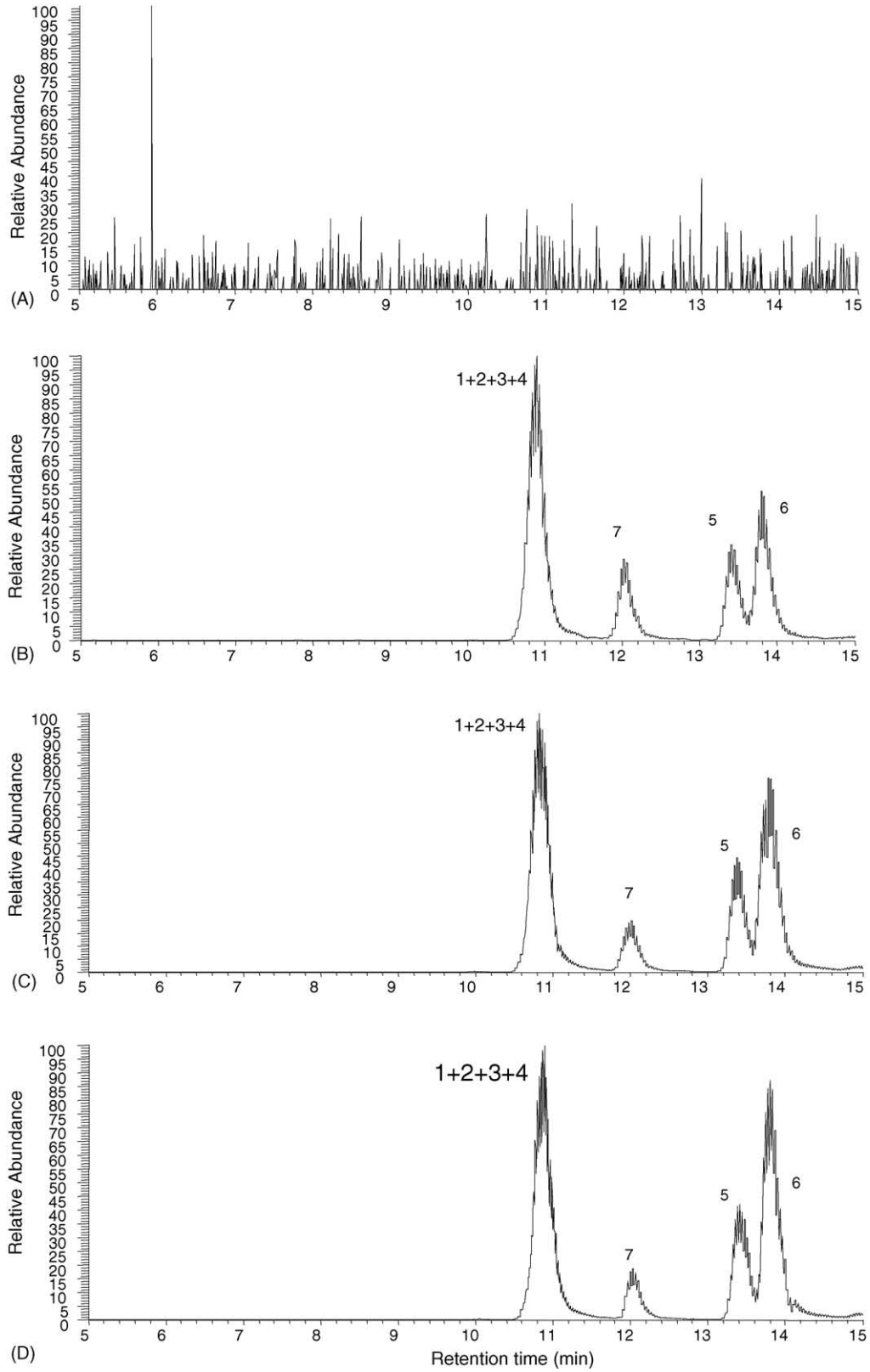


Fig. 3. Reconstructed total ion chromatograms obtained by analysing commercial herbal extracts. Example of one negative sample (A) and all positive samples (B–D). (1) Kavain; (2) dihydrokavain; (3) methysticin; (4) dihydromethysticin; (5) desmethoxyyangonin; (6) yangonin; (7) degradation product of yangonin. The analyte concentrations are given in Table 4.

Table 4
Total kava lactone content and relative amounts of individual compounds in the 'positive' commercial products

Product	Total kava lactones (mg/g)	Kavain[a]	Dihydrokavain[a]	Methysticin[a]	Dihydromethysticin[a]	Yangonin[a]	Desmethoxyyangonin[a]
B	79	21	20	11	15	25	8
C	95	25	23	10	12	24	6
D	8	19	25	11	19	23	5

[a] Expressed as percentage of the total kava lactone content.

3.5. Selectivity of the method

For the detection of residues of contaminants or other undesired compounds, more ions are usually selected. For positive identification also the relative abundance of the ions is monitored. However, in the case of kava lactones, it seems unlikely that one pure compound would be added to herbal preparations, as these compounds are very difficult to obtain pure and are very expensive. Instead, the presence of a natural extract with several lactones is expected. For routine analysis, the monitoring of at least one fragmentation reaction per compound is considered sufficiently selective, if an extra criterion is used for the minimum number of compounds which should be present in a sample for a positive result. In our procedure, a sample is considered positive for kava extract if more than three of the kava lactones are detected by the present procedure. If needed, extra confirmation can be obtained by re-analysing the sample and monitoring more than one transition per compound or use standard addition of a pure standard or a well-characterised kava extract.

3.6. Analysis of food supplements

Ten herbal products were analysed. Kava lactones were found in only three products all purchased before the market restrictions. No kava lactones were found in products purchased after the market restrictions. Chromatograms of one negative and the three positive samples are shown in Fig. 3.

It should be noted that *cis*-yangonin (compound 7) is present in these samples even in fresh extracts (Fig. 3). This suggests that it is also present in the powdered extracts. The calculated concentrations are shown in Table 4. Although there is a large difference in the total content of kava lactones, the relative intensities of the individual compounds differ only slightly. Two products contain almost pure kava extract, the third product contains only 8 mg of lactones per gram. These results are in agreement with the specification of the products.

4. Conclusions

A method for the determination of kava lactones in pure kava extracts and herbal mixtures was developed using LC–APCI-MS/MS. The method can be applied to both quality control measurements of pure extracts used in the manufacture of products for medicinal use and for quality control of products sold as food supplements in which the use of kava is prohibited in most European countries. A very generic approach was selected, so that the analysis can be extended to other bioactive compounds often present in herbal mixtures (e.g. ephedrine, pseudoephedrine).

Analysis of commercial food supplements showed that none of these contained kava extract after its use has been restricted to medicinal products.

Formation of a degradation product of yangonin was observed when a standard solution was stored for several days. We propose the degradation product to be *cis*-yangonin. This compound is also present in the commercial powdered extracts of kava roots and herbal mixtures containing this type of extracts. To our knowledge, this is the first time the presence of *cis*-yangonin is reported in kava extracts.

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