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Chromatographic hair analysis for natural kavalactones and their metabolites. A preliminary study

Dosages capillaires des kavalactones naturelles et de leurs métabolites : étude préliminaire

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Abstract – Purpose: Kava is a traditional Pacific beverage made from the root of *Piper methysticum*. It is mainly used for its sedative properties due to lipophilic lactones called kavalactones. Kava action mechanisms include cell membrane stabilisation, inhibition of intracellular Ca^{2+} increase and enzyme inactivation. Chronic or heavy consumption of kava is responsible for the skin taking on a scaly aspect. Biologically, an isolated increase in serum gamma-glutamyltransferase is noted. "Kava bars" or "nakamals" are numerous in the Pacific. In northern Australia, the use of kava is regulated in order to fight against the abuse of this drink. In metropolitan France, we note the presence of some kava bars in several cities. In New Caledonia, in ten years (December 2002–May 2013), we identified twenty-six cases where kavalactones were found in the blood of perpetrators or victims of fatal accidents (resulting in nineteen deaths and twenty-one people seriously injured), suicides and attacks. In addition, in nine cases of sudden unexplained deaths, significant concentrations of kavalactones were found in the blood of the victims. These figures, however, are understated as toxicological screening was not done systematically. As all toxicological laboratories are not identically equipped, we developed three simple and sensitive methods for the determination of kavalactones in human hair using HPLC-DAD, LC-MS/MS and GC/TOF-MS. **Methods:** Hair samples were collected from nine people of different origins (Caucasian, Melanesian, Indonesian and African). Kava consumption varied among these people (from a single oral dose of a medication sold on the internet, to a daily intake of several units of the kava beverage). **Results:** Using HPLC-DAD, the concentrations of kavalactones in human hair samples ranged between 0.2 and 25 ng/mg for kavain, 0.5 and 34 ng/mg for 7,8-dihydrokavain, 0.7 and 8 ng/mg for yangonin, 1 and 14 ng/mg for 5,6-dehydrokavain (=desmethyoxyyangonin) and 0.9 and 6 ng/mg for the metabolite 12-hydroxy-5,6-dehydrokavain. Methysticin, 7,8-dihydromethysticin and the metabolite 11-hydroxy-5,6-dehydrokavain were detected but not quantified. Additionally, 12-hydroxykavain and 12-hydroxy-7,8-dihydrokavain were detected by LC-MS/MS in one case. General screening for other drugs as well as confirmation of the HPLC-DAD results was performed by GC/TOF-MS. **Conclusions:** The results of this pilot study indicate that kavalactones (kavain, 7,8-dihydrokavain, methysticin, 7,8-dihydromethysticin, 5,6-dehydrokavain (=desmethyoxyyangonin) and yangonin) accumulate in the keratin matrix of hair and can provide an easily applicable system for assessing chronic consumption of kava. This preliminary study must continue on a larger number of subjects using GC/TOF-MS and LC-MS/MS in order to conduct a comparative analysis among the three methods.

Key words: Kavalactones, hair, HPLC-DAD, LC-MS/MS, GC/TOF-MS

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Résumé – Objectifs : Le kava est une boisson traditionnelle du Pacifique, fabriquée à partir de la racine de *Piper methysticum*. Il est principalement utilisé pour ses propriétés sédatives dues à des lactones lipophiles appelées kavalactones. Les mécanismes d'action du kava comprennent un effet stabilisateur de membrane cellulaire, une inhibition de l'augmentation des concentrations intracellulaires de Ca^{2+} et l'inactivation d'enzymes. Après une consommation chronique et importante, le kava est responsable d'un aspect de peau écailleuse. Biologiquement, on note une augmentation isolée de gamma-glutamyltransférase sérique. Les bars à kava ou « nakamals » sont nombreux dans le Pacifique. Dans le nord de l'Australie, l'utilisation du kava est réglementée afin de lutter contre les abus de la boisson. En France métropolitaine, on note la présence de quelques bars à kava dans plusieurs villes. En Nouvelle-Calédonie, en dix ans (décembre 2002–mai 2013), nous avons identifié vingt-six cas où des kavalactones ont été trouvées dans le sang d'auteurs ou de victimes d'accidents mortels (ayant causé dix-neuf morts et vingt et un blessés graves), de suicides ou d'agressions. En outre, dans neufs cas de morts subites inexpliquées, des concentrations importantes de kavalactones ont été trouvées dans le sang des victimes. Ces chiffres sont toutefois sous-évalués, le dépistage toxicologique n'ayant pas été réalisé de façon systématique. **Méthodes :** Tous les laboratoires de toxicologie n'étant pas équipés de façon identique, nous avons développé trois méthodes simples et sensibles pour la détermination de kavalactones dans les cheveux par HPLC-DAD, LC-MS/MS et GC/TOF-MS. Les échantillons de cheveux ont été prélevés sur neuf personnes différentes, et de différentes origines ethniques (mélanésienne, indonésienne, européenne et africaine). La consommation de kava variait entre ces personnes (d'une dose orale unique d'un médicament vendu sur Internet, à l'absorption quotidienne de plusieurs unités de boisson kava). **Résultats :** Déterminées par HPLC-DAD, les concentrations de kavalactones dans des échantillons de cheveux variaient de 0,2 à 25 ng/mg pour la kavaïne, de 0,5 à 34 ng/mg pour la 7,8-dihydrokavaïne, de 0,7 à 8 ng/mg pour la yangonine, de 1 et 14 ng/mg pour la 5,6-déhydrokavaïne (= desméthyoxyyangonine) et de 0,9 à 6 ng/mg pour le métabolite 12-hydroxy-5,6-déhydrokavaïne. La méthysticine, la 7,8-dihydrométhysticine et le métabolite 11-hydroxy-5,6-déhydrokavaïne ont été détectés mais non quantifiés. En outre, la 12-hydroxykavaïne et la 12-hydroxy-7,8-dihydrokavaïne ont été détectés par LC-MS/MS dans un cas. Le criblage toxicologique des autres drogues ainsi que la confirmation des résultats obtenus par HPLC-DAD a été réalisé par GC/TOF-MS. **Conclusions :** Les résultats de cette étude pilote indiquent que les kavalactones (kavaïne, 7,8-dihydrokavaïne, méthysticine, 7,8-dihydrométhysticine, 5,6-déhydrokavaïne (= desméthoxyyangonine) et yangonine) s'accumulent dans la kératine des cheveux et peuvent fournir une méthode facilement réalisable pour évaluer l'importance d'une consommation de kava. Cette étude préliminaire se poursuit sur un plus grand nombre de sujets afin de pouvoir réaliser une analyse comparative entre les trois méthodes.

Mots clés : Kavalactones, cheveux, HPLC-DAD, LC-MS/MS, GC/TOF-MS

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1 Introduction

Piper methysticum is one of the most important crops in its area of circulation (*e.g.* the islands of the South Pacific such as Oceania) and is cultivated with considerable care. The roots of the kava plant are crushed or chewed and doused with cold water. This method of preparation gives only a very incomplete extraction of the water-soluble kavapyrones (=kavalactones). For this reason, pharmaceutical products of *Piper methysticum* are predominantly prepared in the form of ethanolic extracts. While extracts of *Piper methysticum* contain only the (+) form of kavain, the synthetic preparations of kavain as a pharmaceutical product are distributed as two enantiomers [1].

Kava has also become a drug of abuse amongst some aboriginal communities, due to its sedative and relaxant properties. Acute kava misuse leads to euphoria, sedation, muscle weakness, ataxia and ichthyosis [2–7]. In a few states or countries, as in Australia, regulatory measures exist concerning kava importation and kava markets, but to our knowledge, neither kava nor kavain or other kavalactones are classified as psychotropic products or as narcotics.

Kava is imported mainly from Fiji, Vanuatu and Hawaii. Aqueous extraction of the commercially dried plant material is used for the preparation of the kava beverage [7, 8]. The main requirements for beverage preparation are the kava stock, a bowl, cups, a strainer and water. Depending on the nature of the occasion, kava is available in the form of fine roots, or roots and stems, which are then reduced to fine particles, or commercially prepared powder may be used [7]. Nowadays, for instance in New Caledonian and Fijian kava bars, kava is prepared by crushing the dry roots of *Piper methysticum* in tap water, in a basin, then filtered and drunk. Several studies about the detection and identification of kavalactones and their metabolites in urine have been published [9–12]. Kavain and its main metabolites have also been detected in blood [13–16].

Kava action mechanisms include cell membrane stabilisation, inhibition of intracellular Ca^{2+} increase and enzyme inactivation. After chronic or heavy consumption, kava is responsible for the skin taking on a scaly aspect. Biologically, an isolated increase in serum gamma-glutamyltransferase is noted. "Kava bars" or "nakamals" are numerous in the Pacific. In northern Australia, the use of kava is regulated in order to fight against the abuse of the drink. In metropolitan France, we note the presence of some kava bars in several cities. In New Caledonia, in ten years (December 2002–May 2013), we identified twenty-six cases where kavalactones were found in the blood of perpetrators or victims of fatal accidents (resulting in nineteen deaths and twenty-one seriously injured), suicides and attacks. In addition, in nine cases of sudden unexplained deaths, significant concentrations of kavalactones were found

in the blood of the victims. These figures, however, are understated as toxicological screening was not done systematically.

Fifteen lactones have been isolated from kava roots [17–21]; ten of these have been defined as major components of kava and were fully identified (kavain, 5,6-dehydrokavain, 7,8-dihydrokavain, methysticin, 5,6-dehydromethysticin, 7,8-dihydromethysticin, yangonin, 5,6-dihydroyangonin, 7,8-dihydroyangonin and tetrahydroyangonin). Five other compounds are minor compounds in the root stock (5α-hydroxykavain, 12-methoxyyangonin, 12-hydroxyyangonin, 12-methoxy-11-hydroxy-5,6-dehydrokavain and 13-methoxyyangonin: Figures 1A and 1B). The total amount of the kavalactones or kavapyrones in kava roots or rhizomes is 3 to 5% (according to the literature, the roots should contain at least 3.5% of kavain). In the dry root of the kava plant, the percentages of kavalactones were: 1 to 2% kavain, 0.6 to 1% 7,8-dihydrokavain, 1.2 to 2% methysticin, 0.5 to 0.8% 7,8-dihydromethysticin and 0.9 to 1.7% yangonin and 5,6-dehydrokavain (=desmethoxyyangonin) [22–25].

To our knowledge, the pharmacokinetic and pharmacodynamic parameters of natural kavalactones have not been determined before. For kavain, these parameters have been established using 200 mg synthetic D,L-kavain [16]: the peak plasma concentration is reached in about 1.8 h, the kavain elimination half-life is about 9 h and the distribution phase lasts 3 to 5 h [16]. However, kavalactones administered *per os* have a weak digestive absorption, especially if each kavalactone is administered separately, which is not the case in the kava beverage [8]. In our opinion, these results should not be extrapolated to the natural D-kavain. For this reason, we did our own experiments using HPLC-DAD and GC-MS [8]. After a 36-hour wash-out, a 42-year-old male chronic kava drinker ingested 145 mL (about three cups) of a kava beverage of known composition. For this study, the amounts of kavalactones ingested were: kavain: 255 mg, 7,8-dihydrokavain: 427 mg, 7,8-dihydromethysticin + methysticin: 535 mg, yangonin: 268 mg, desmethoxyyangonin (=5,6-dehydrokavain): 479 mg. Peak plasma concentrations of D-kavain appeared in 45 min, reflecting the rapid installation of kava's psychotropic effects; kavain t 1/2 was about 2.5 h and after 13 h, kavalactones were still found in plasma [8]. We also demonstrated the persistence of kavalactones for up to 24 h in the plasma of a kava drinker with an abusive mode of consumption, which shows a possible accumulation of these products. In urine, kavalactones and their metabolites have been detected for up to 48 h. In our study, the distribution volume was not determined.

Regarding toxicological studies, the same remark arises: the action of synthetic D,L-kavain might differ from the natural D-kavain [8]. Experiments that demonstrated kavain's strong liver toxicity used synthetic kavain made of L-kavain or D,L-kavain [8,16]; some used an intravenous model [8]. In Europe, accidents have occurred with products containing synthetic kavain or kava extracts enriched with synthetic kavain [8]. To our knowledge, in Pacific countries where the kava beverage is drunk in large amounts, there are no reports of liver failure following acute or chronic kava intoxication. In a retrospective study, over ten years in New Caledonia, we reported only two cases of kava-related hepatitis and concluded that the traditional beverage could also be responsible for rare cases of hepatitis [8]. In addition, all cases of liver toxicity (with beverages or medications) occurred at low kava dosages – compared with what is currently traditionally consumed – and with new kava consumers. This point indicates an idiosyncratic mechanism rather than a dose-related toxicity. The second important point is that intravenous kavain administration does not compare with a progressive oral administration of a kavalactone mixture [8].

In recent years, human hair has received considerable attention as a toxicological specimen for evidence of chronic drug use in both clinical and forensic investigations and is now being recognised as the third fundamental biological specimen for drug testing after urine and blood [26].

To the best of our knowledge, identification and quantification of other kavalactones, except kavain in human hair, have never been published before. The only available data about kavalactones in hair was presented by a French team where kavain was determined in a segment of hair obtained from an occasional consumer, which revealed concentrations of 418 and 1708 pg/mg in head and pubic hair, respectively [27]. Generally, hair concentrations of different drugs attained after similar dosages can differ by orders of magnitude because of the influence on drug incorporation, such as the lipophilicity and basicity of the drugs, content of hair melanin and differences in hair growth [26,28–30].

In this paper, we investigated the presence of the kavalactones and some of their metabolites in hair samples of nine people after kava consumption. This is the first report on the detection of natural kavalactones in hair after kava beverage consumption. The analysis proved to be a reliable tool for the retrospective detection of chronic kava abuse in forensic toxicology and in addictology.

2 Material and methods

2.1 Reagents

All compounds were of analytical grade: methanol, ethanol, acetone, petroleum benzene, acetonitrile, disodium hydrogen phosphate, potassium dihydrogenophosphate, anhydrous sodium sulphate, sodium hydroxide, water for chromatography (Merck; Darmstadt, Germany), diethylether (Fluka; Neu-Ulm, Germany), Bakerbond spe™ columns (J.T. Baker; Deventer, Holland), deionised water (<0.1 µS from a cartridge deioniser, Memtech; Moorenweis, Germany), gradient grade acetonitrile, ammonium formate (Sigma; Deisenhofen, Germany) and formic acid (Merck; Darmstadt, Germany).

Kavain, 12-hydroxy-5,6-dehydrokavain, 12-hydroxykavain, 12-hydroxy-7,8-dihydrokavain, 6-phenyl-5-hexen-2,4-dion (Klinge Pharm Ltd.; Munich, Germany). 5,6-dehydrokavain (Analyticon; Berlin Wedding, Germany), 7,8-dihydrokavain, yangonin and methysticin (Dr Daniel Duhet, IRD; Laboratoire des Substances Naturelles Terrestres, Promenade Roger Laroque, BP A5, 98 848 Nouméa, Nouvelle-Calédonie); brotizolam (Promochem; Wesel, Germany).

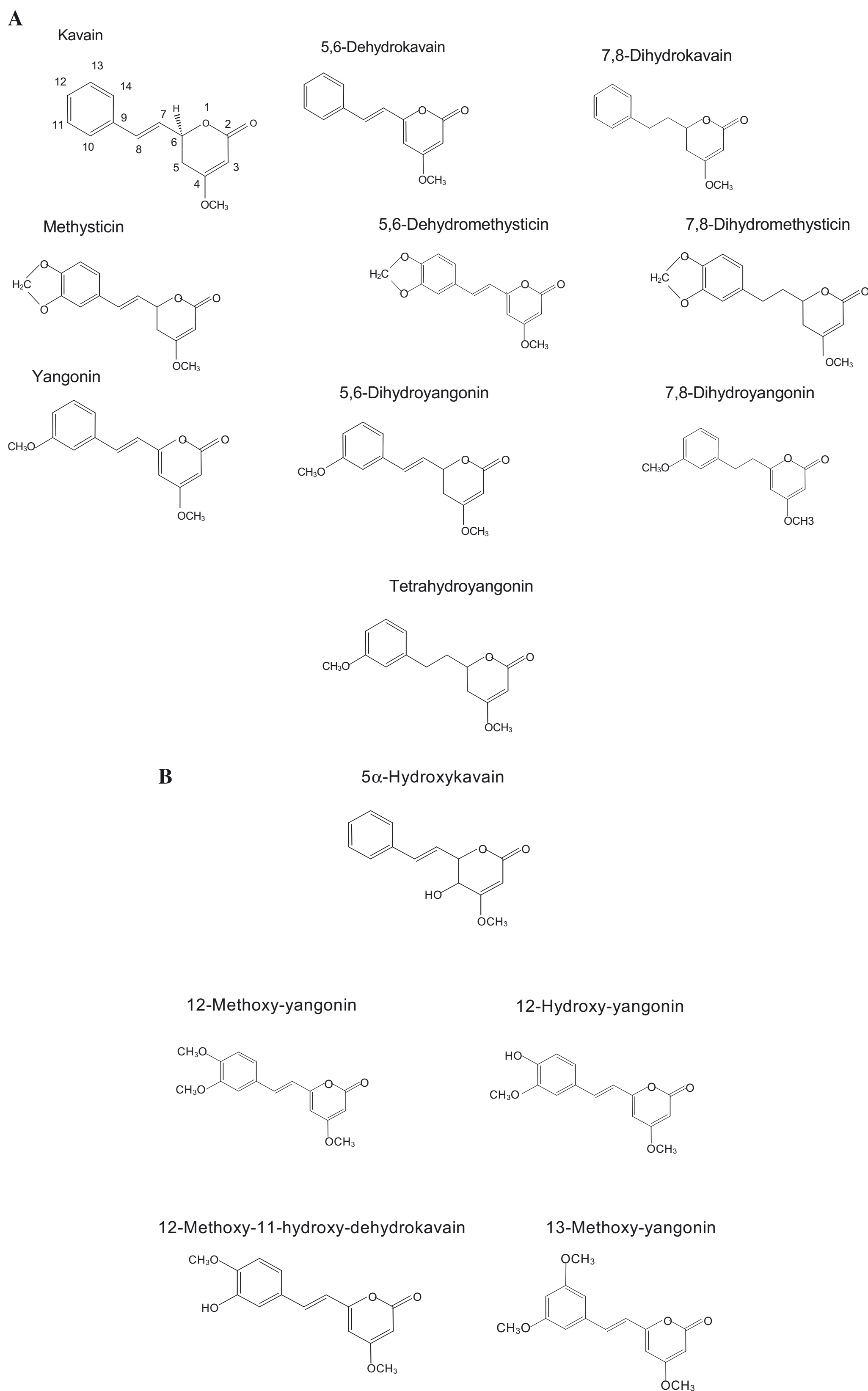

Fig. 1. (A) Major kavalactones. (B) Minor kavalactones.

Table I. Database about the nine people and their kava consumption behaviour.

Patient No.	Sex	Age in years	Length & weight	Origins	Segment used (cm)	Hair weight (mg)	Hair type	Kava consumption (personal reporting)
(1)	M	55	170/75	Caucasian	3	21	sm/bl	4 cups/day since 1994
(2)	M	33	170/80	Melanesian	4	102	cu/bl	Big drinker (ichthyosis)
(3)	M	30	169/55	Indonesian	0 to 6	280	sm/bl	0.6 L/day
(4)	F	23	165/62	Caucasian	0 to 6	342	sm/br	0.4 L/day, stopped for 1 year and re-introduced kava 1 month ago
(5)	M	35	165/75	Melanesian	6	97	sm/bl	1 L/day, more than 10 years
(6)	M	42	–	Caucasian	4	253	sm/br	4 cups/day
(7)	M	28	–	Caucasian	0 to 4	113	sm/br	Psychiatric patient (kava, cannabis, cocaine and MDMA)
(8)	M	50	–	Caucasian	0 to 4	118	sm/bl + gr	Only one consumption of one cup, hair collection after one month
(9)	M	36	187/104	African	0 to 4	174	cu/bl	1 L (contains 90 g) of kava at once, hair collected 2 months later

Hair description: sm = smooth; cu = curly; bl = black; br = brown; gr = grey.

2.2 Standard preparation

Stock solutions of the reference substances kavain, 7,8-dihydrokavain, 5,6-dehydrokavain, yangonin, methysticin, 12-hydroxykavain, 12-hydroxy-7,8-dihydrokavain and 12-hydroxy-5,6-dehydrokavain were prepared in methanol at a concentration of 0.1% (1 mg/mL) and stored at 4 °C. Further dilutions were done when needed.

2.3 Biological material (hair sampling)

Hair samples (cases 1-7) were collected at the Central Hospital of Nouméa, New Caledonia, France; cases 8 and 9 were self-trials by one of the authors and a collaborator of the study (Table I). Hair samples were collected and stored in a dark and dry place at room temperature until analysis.

2.4 Internal standards and buffers

Brotizolam in methanol 0.01% (0.1 mg/mL) was used as an internal standard for HPLC.

Buffer pH 9: 1.78 g of disodium hydrogenophosphate (Na_2HPO_4) was dissolved in 100 mL water.

Buffer for HPLC elution system: 156 g acetonitrile was mixed with 344 g buffer (4.8 g $H_3PO_4$85% and 6.66 g KH_2PO_4) and was completed with water to 1 L, pH 2.3.

2.5 Instruments

2.5.1 High-performance liquid chromatography (HPLC-DAD)

The analyses were performed with a Waters 2690 separation module fitted with a Waters 996 PDA Detector. Separation was carried out with a reversed-phase LiChrospher 60, RP-select B column 250 mm × 4.0 mm ID, particle size 5 µm, (Merck; Darmstadt, Germany) with isocratic conditions (pH 2.3, (acetonitrile 31.2%, and phosphate buffer 68.8%, w/w)) at a flow rate of 1 mL/min; the column temperature was set at 27 °C. Chromatograms were recorded at 190–420 nm with a resolution of 1.2 nm. The reference wave length 246 nm was set for kavain, 238 nm for 7,8-dihydrokavin, methysticin and 7,8-dihydromethysticin, 350 nm for yangonin, 5,6-dehydrokavain and 12-hydroxy-5,6-dehydrokavain, 226 nm for 12-hydroxy-7,8-dihydrokavain, and 262 nm for 12-hydroxykavain.

2.5.2 High-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS)

A HTC PAL autosampler (Chromtech GmbH; Idstein, Germany) fitted with a Hamilton 100-µL syringe (Microliter Syringes, Chromtech GmbH; Idstein, Germany) and two LC10AD pumps (Shimadzu; Duisburg, Germany) were used. The chromatographic separation was performed using a Luna RP-C18 analytical column (150 mm × 2 mm I.D., 3.0 µm, Phenomenex; Aschaffenburg, Germany), thermostated at 40 °C. The mobile phase consisted of solvent A (0.1% formic acid, 1 mM ammonium formate, pH 2.7) and solvent B (acetonitrile/0.1% formic acid, 1 mM ammonium formate, 95:5, v/v). The isocratic (36% B) LC method took 28 min, the flow rate was 250 µL/min and the injection volume 20 µL.

The total eluate from the LC column was directed into the TurboIonSpray source (electrospray ionisation (ESI)) of a Sciex API 365 triple quadrupole mass spectrometer without splitting. The needle voltage was set to 5.25 kV and the nebuliser gas was set to 3 L.min^{-1}. Declustering potential was 20 V, focusing potential was 230 V, entrance potential was 10 V, and collision exit potential was 15 V. The collision energy for each transition is shown in Tables II and III. Ultra-pure nitrogen was used as the collision cell gas. The TurboIonSpray heater

Table II. Kavalactones in hair sample of case 2 as an example, detected by GC/TOF-MS.

Compound	$[M]^+$	[m/z]	Retention time in sec.
7,8-dihydrokavain	232	232 (25%), 200 (30%), 173 (8%), 127 (100%), 91 (60%), 68 (45%)	858
Kavain	230	230 (35%), 202 (45%), 98 (90%), 68 (100%)	901
Tetrahydroyangonin	262	262 (15%), 147 (35%), 121 (100%), 230 (2%), 163 (5%)	966
7,8-dihydromethysticin	276	276 (35%), 135 (100%), 161 (20%), 91 (15%), 2174 (3%)	1013
Methysticin	274	274 (45%), 246 (5%), 207 (7%), 175 (20%), 148 (100%), 135 (85%)	1058

Table III. Protonated molecular $[M+H]^+$, MRM transitions and collision energy.

Compound	$[M+H]^+$	Transitions [m/z]	Collision energy [eV]
12-hydroxykavain	247	247 → 109	20
		247 → 131	20
Methysticin	275	275 → 159	20
		275 → 103	35
Tetrahydroyangonin	263	263 → 121	20
		263 → 147	20
7,8-dihydrokavain	233	233 → 117	20
		233 → 155	20
Yangonin	259	259 → 161	20
		259 → 133	35

was set at 300 °C. MS/MS of compounds and sample extracts was performed in triple-quadruple mode (MRM) using a dwell time of 200 ms.

2.5.3 Gas chromatography time-of-flight mass spectrometry (GC/TOF-MS)

GC analysis was carried out on a Model 6890 GC (Agilent Technologies; Palo Alto, CA, USA) instrument which was equipped with an OPTIC 3 injector (ATAS GL International; Veldhoven, The Netherlands). The column: HP-5 MS, 30 m length, ID 0.25 mm (0.25 µm film thickness); carrier gas: ultrapure helium (pressure 70 kPa); split/purge off time: 2 min; injector temperature: 270 °C; transfer line temperature: 280 °C; temperature programme: initial temperature 40 °C for 2 min, 40 °C/min to 100 °C for 3.5 min, 11 °C/min to 300 °C for 12.75 min (total run time: 33.5 min). Detection was carried out on a PEGASUS III time-of-flight mass spectrometer (LECO, Instrumente GmbH; Mönchengladbach, Germany) controlled by a ChromaTOF sofware package (LECO) for data acquisition, data processing and peak deconvolution.

2.6 Hair sample preparation and extraction procedure

In headspace glasses, hair samples were washed for 5 min in 5 mL of HPLC water, acetone and finally, with petroleum benzene. After drying (overnight) the hair samples were cut into small pieces of about 1 mm. The entire capillary segment was used and according to the sample weights (Table I), 3 or 6 mL of methanol were added, and the headspace glasses were tightly closed and placed into an ultrasonic water bath for 5 h (caution: water temperature should not exceed 60 °C).

The methanol layer was collected and filtered using the described filtration columns and the methanol layer was evaporated under a stream of nitrogen until dry. The residue was collected in 0.1 mL buffer (pH 9) and 0.7 mL HPLC water.

The aqueous phase (0.8 mL) was mixed with 10 µL (brotizolam 0.01%) as an internal standard. The samples were extracted with 1 mL of dichloromethane:diethylether 7:3, v/v [31], vortexed for 1 min and centrifuged at 14 000 rpm for 5 min at 10 °C. The lower organic phase was dried with sodium sulphate, centrifuged and transferred into a high recovery glass vial. The organic layer was evaporated until dry (N_2 stream), and the extract residue was reconstituted in methanol (according to hair weight, *i.e.* mg hair/µL methanol). 10 µL were analysed by HPLC-DAD.

An aliquot of 20 µL of hair extract was diluted in 400 µL methanol. 1 µL of the extract was injected into the GC/TOF-MS. Another aliquot of the extract was evaporated until dryness (N_2 stream), reconstituted in 100 µL solvent A:B (8:2, v/v), and 20 µL of the extract were injected into the LC-MS/MS system.

3 Results and discussion

For the purposes of this work, the investigation of the migration of kavalactones and their metabolites into the hair matrix, the detection of these compounds was carried out with hair samples of different kava consumers. The given data (Table I) about the consumers' behaviour was based on the consumers self-reporting. Kavain, 7,8-dihydrokavain, methysticin and yangonin are known to be stable in methanol [10–12]. Therefore, the methanolic extraction of the hair matrix and subsequent fluid-fluid extraction were suitable.

The analysis of the hair samples of eight people revealed the presence of kavain, 7,8-dihydrokavain, 5,6-dehydrokavain (=desmethoxyyangonin), yangonin, tetrahydroyangonin, methysticin, 7,8-dihydromethysticin and 12-hydroxy-5,6-dehydrokavain, 12-hydroxy-7,8-dihydrokavain, and 12-hydroxykavain as known metabolites. (Figures 2a and 2b and Figures 3 and 4).

3.1 HPLC-DAD

Because of numerous double bonds and especially their sequence (alternating double and single bonds) and pi-electrons,

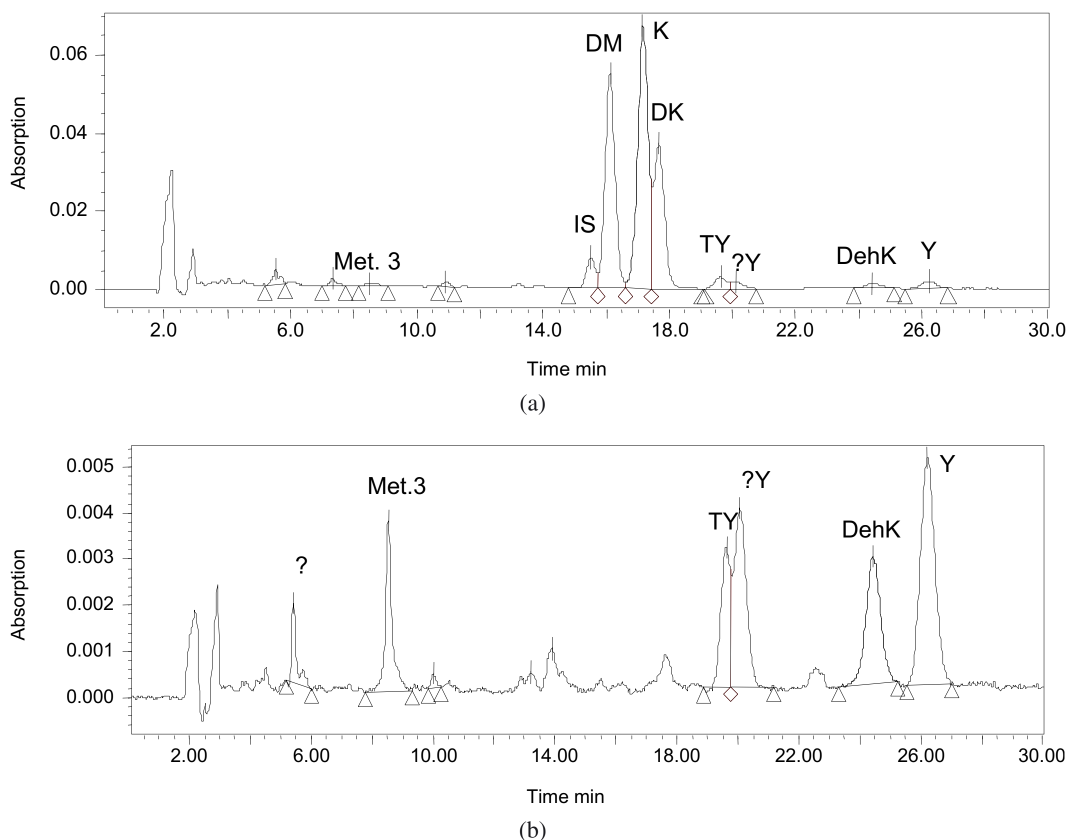

Fig. 2. (a) HPLC chromatogram at 246 nm of a hair sample taken after more than 10 years of regular kava consumption: Met 3: 12-hydroxy-5,6-dehydrokavain *(RT: 8.5 min)*, IS: internal standard brotizolam (*RT: 15.3 min*), DM: 7,8-dihydromethysticin *(RT: 16.3)*, K: kavain *(RT: 17.2)*, DK: 7,8-dihydrokavain *(RT: 17.7)*, TY: tetrahydroyangonin *(RT: 19.8)*, ?Y: UV spectrum related to yangonin *(RT: 20.07 min)*, Dehk: 5,6-dehydrokavain *(RT: 24.4)* and Y: yangonin *(RT: 26.4)*. (b) HPLC chromatogram at 350 nm of a hair sample taken after more than 10 years of regular kava consumption: Met. 3: 12-hydroxy-5,6-dehydrokavain *(RT: 8.5 min)*, TY: tetrahydroyangonin *(RT: 19.8)*,?Y: UV spectrum related to yangonin *(RT: 20.07 min)*, Dehk: 5,6-dehydrokavain *(RT: 24.4)* and Y: yangonin *(RT: 26.4)*.

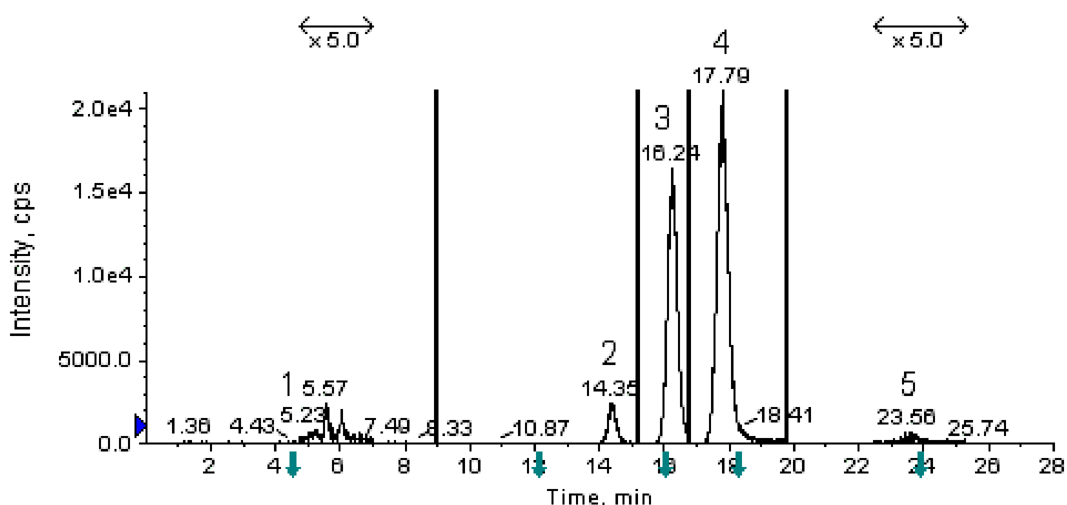

Fig. 3. LC-MS/MS total ion chromatogram with MRM mode: 1: traces of 12-hydroxykavain (*RT: 5.23 min*), 2: methysticin (*RT: 14.35 min*); 3: tetrahydroyangonin (*RT: 16.24 min*), 4: 7,8-dihydrokavain (*RT: 17.79 min*) and 5: yangonin (*RT: 23.56 min*).

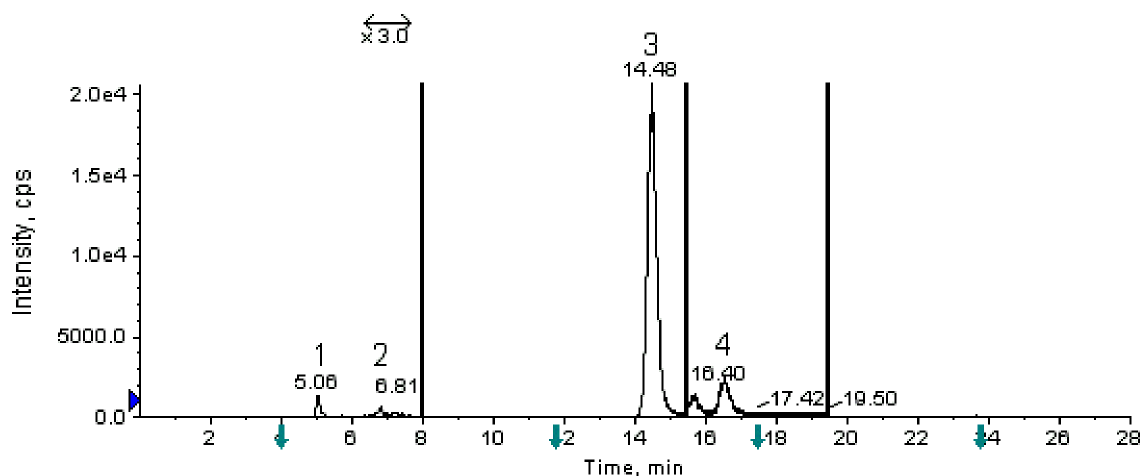

Fig. 4. LC-MS/MS total ion chromatogram with MRM mode: 1: 12-hydroxy-7,8-dihydrokavain (*RT: 5.06 min*), 2: 12-hydroxy-5,6-dehydrokavain (*RT: 6.81 min*), 3: 7,8 dihydromethysticin (*RT: 14.48 min*) and 4: kavain (*RT: 16.40 min*).

the molar extinctions of the kavalactones are very high and make kavalactones easy to detect by DAD. The method was originally developed and validated for the determination of kavalactones and their metabolites in blood and in urine (16). At the time, validation categories such as linearity, inter- and intra-day precision and the limit of detection were determined using Valistat® software.

The UV spectra and retention times of kavain, 5,6-dehydrokavain, methysticin, yangonin, 12-hydroxykavain, 12-hydroxy-5,6-dehydrokavain and 12-hydroxy-7,8-dihydrokavain were measured and compared with the available reference substances for identification (Figures 5a–5h). Using the HPLC-DAD method, methysticin and 7,8-dihydromethysticin could not be completely separated and consequently could not be quantified.

3.2 GC/TOF-MS

The hair samples were also analysed for general screening using GC/TOF-MS. Kavalactones were detected and identified according to the reference substances available and literature data [9–12, 14, 16, 20, 21]. The qualitative determination of kavalactones in hair was controlled by a ChromaTOF software package (LECO) for data acquisition. The results are summarised in Table II. The co-elution between the extracted hair matrix and the kavalactones, *e.g.* kavain and 7,8-dihydrokavain, is solved using algorithm software which helps in the identification of the unknown analytes buried beneath analytes at high concentrations or below the background of the total ion chromatogram (TIC) (Figure 6).

Kavain and 7,8-dihydrokavain were detected in the hair of eight people even when small hair amounts were available (case 1). Additional 7,8-dihydrokavain, 7,8-dihydromethysticin and tetrahydroyangonin could be identified by GC/TOF-MS according to the literature data [14, 20]. A better separation between methysticin and 7,8-dihydromethysticin was also observed (Table II).

Table IV. Protonated molecular $[M+H]^+$, MRM transitions and collision energy.

Compound	$[M+H]^+$	Transitions [m/z]	Collision energy [eV]
12-hydroxy-7,8-dihydrokavain	249	249 → 133	20
		249 → 107	20
p-hydroxy-5,6-dehydrokavain	245	245 → 217	20
		245 → 147	35
6-phenyl-hexan-2,4-dion	189	189 → 171	20
		189 → 131	20
5,6-dihydroyangonin	261	261 → 173	20
		261 → 201	20
Dihydromethysticin	233	277 → 135	20
		277 → 161	20
Tetrahydroyangonin	263	263 → 121	20
		263 → 147	20
Kavain	231	231 → 147	20
		231 → 115	20
5,6-dehydrokavain	229	229 → 131	35
		229 → 141	35

3.3 LC-MS/MS

The application of the LC-MS/MS method using the different chosen transitions in MRM mode made it possible to identify methysticin and 7,8-dihydromethysticin (Figures 3 and 4). 7,8-dihydrokavain, 7,8-dihydromethysticin and tetrahydroyangonin were also identified by LC-MS/MS according to the literature data [14, 20]. For LC-MS/MS analysis, two transitions per compound were chosen (Tables III and IV). For the determination, the compounds were divided into two groups. For each group, one MS/MS method was created. Both were separated into four windows to obtain a high sensitivity. Analyst 1.3.1 Software (Applied Biosystems; Darmstadt, Germany) was used. MS/MS transitions and parameters are summarised in Tables III and IV.

The quantifications of kavalactones in hair were carried out by calculation of the peak height ratio (substance to internal

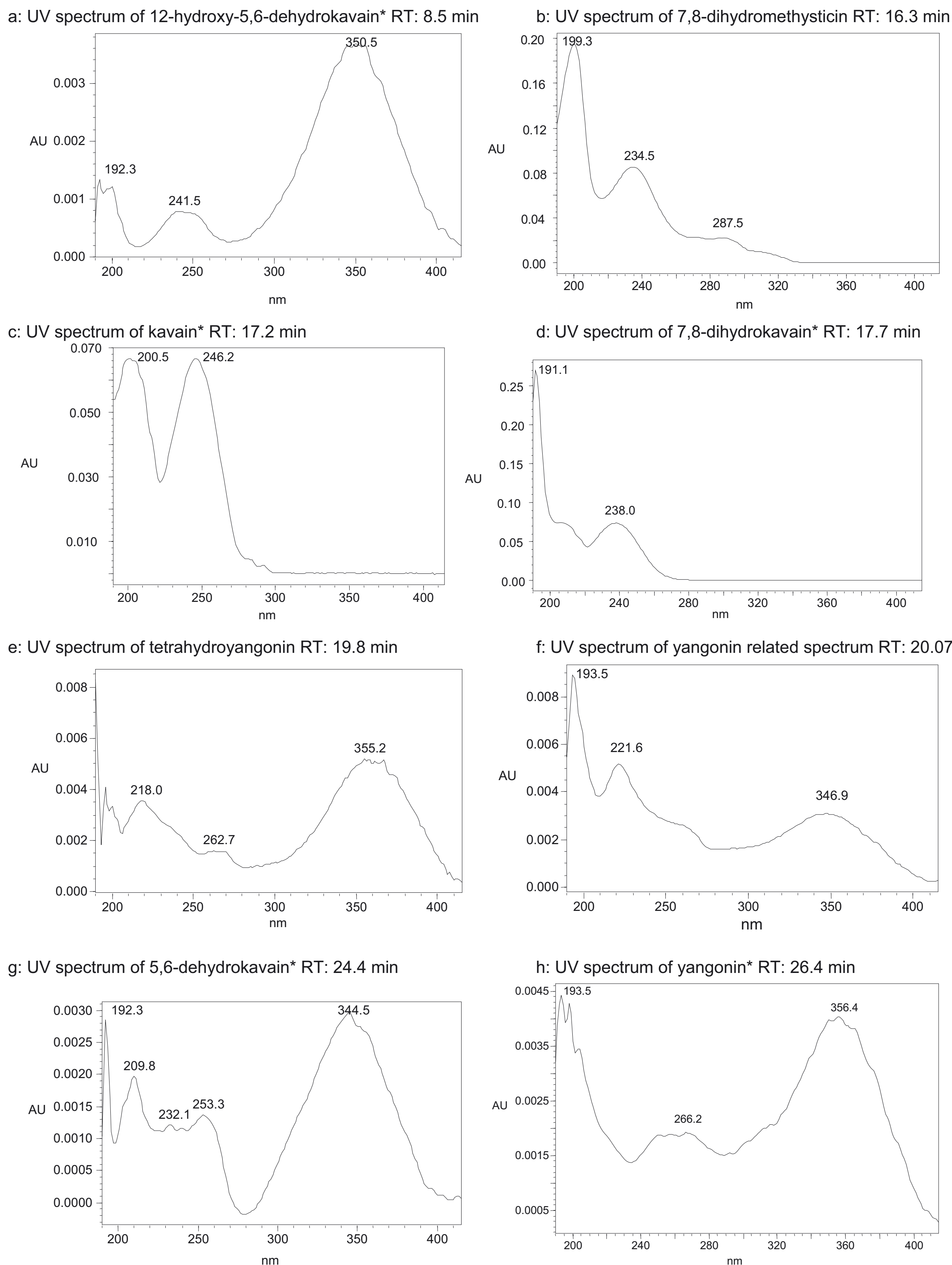

Fig. 5. (a)–(h): UV spectra and retention times (RT) of the major kavalactones and their metabolites detected in the hair samples after 10 years of regular kava consumption.
*: reference substances available.

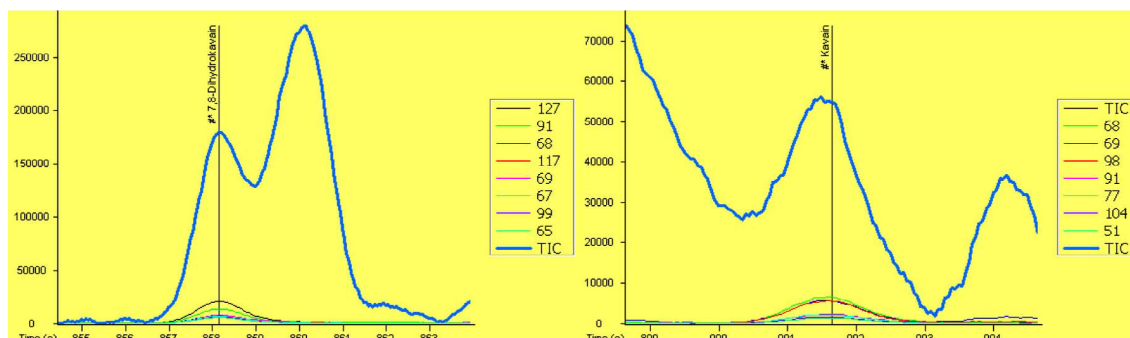

Fig. 6. Kavain and 7,8-dihydrokavain are automatically located beneath the reconstructed total ion chromatogram of the hair sample extract.

Table V. Determination of kavalactones in hair specimens using HPLC-DAD.

Patient	K ng/mg	DK ng/mg	M/DM ng/mg	Y ng/mg	DehK ng/mg	Met 1 ng/mg	Met 2 ng/mg	Met 3 ng/mg	Met 4 ng/mg
(1)	0.5	0.8	neg	2	1	neg	neg	0.9	neg
(2)	5	10	++	1	1	neg	neg	4	neg
(3)	2	2.6	+	2	3.0	neg	neg	6	+
(4)	0.3	0.7	neg	neg	neg	neg	neg	neg	neg
(5)	25	34	+ + ++	8	14	++	++	2.5	+
(6)	0.2	0.5	+	neg	neg	neg	neg	1.5	neg
(7)	11	14	+ + +	3	5	neg	neg	1	neg
(8)	0.4	0.6	+	0.7	neg	neg	neg	3	neg
(9)	neg	neg	neg	neg	neg	neg	neg	neg	neg

K: kavain; DK: 7,8-dihydrokavain; M/DM: methysticin and/or 7,8-dihydromethysticin detected but not quantified; Y: yangonin; DehK: 5,6-dehydrokavain (=desmethoxyyangonin); Met 1: 12-hydroxykavain; Met 2: 12-hydroxy-7,8-dihydrokavain; Met 1 and Met 2 detected only by LC-MS/MS; Met 3: 12-hydroxy-5,6-dehydrokavain, Met 4: 11-hydroxy-5,6-dehydrokavain; (+: detected but not quantified); neg: not detected.

standard) of each kavalactone detected to the peak height ratio of the available reference substances.

3.4 Kavalactone hair concentrations according to kava consumption

Probably due to different modes of supply of raw material by kava bars and also probably by different modes of preparation of the beverage (more or less water), in spite of an equal volume of kava consumed in cases 1, 5 and 6, no similarities were observed in the detected amounts of kavalactones (Table V). Kavain, 7,8-dihydrokavain, yangonin and 5,6-dehydrokavain were detected with the highest concentration in case 5 (Table V). The metabolite 12-hydroxy-5,6-dehydrokavain (Met. 3) was found in all of the cases except cases 4 and 9 (Table V). Case 3 showed, on one hand, the highest concentration of this metabolite and, on the other hand, parent kavalactones were lower compared with cases 5 and 7 (Table V). In case 9, the hair sample was collected two months after oral administration of a single dose (1 L) of kava beverage. Due to the detection limit of HPLC-DAD, no kavalactone or kavalactone metabolite was detected in case 9 (Table V).

The concentrations of kavalactones varied widely over a range between 0.2 and 25 ng/mg for kavain, 0.5 and 34 ng/mg for 7,8-dihydrokavain, 0.7 and 8 ng/mg for yangonin, 1 and 14 ng/mg for 5,6-dehydrokavain (=desmethoxyyangonin) and 0.9 and 6 ng/mg for the metabolite 12-hydroxy-5,6-dehydrokavain. Methysticin and 7,8-dihydromethysticin were semi-quantified (Table V).

The highest concentrations of kavalactones were detected in the hair sample of case 5, who had consumed one litre of kava beverage daily over the past 10 years. Case 4, who stopped for one year and recommenced consumption one month ago, showed the lowest concentrations of all the subjects. No kavalactone or metabolite was detected after a single oral dose in case 9, but the second self-medicated case (8) showed low concentrations of kavain and 7,8-dihydrokavain and a relatively high concentration of 12-hydroxy-5,6-dehydrokavain.

4 Conclusion

Kava is a traditional Pacific beverage with psychotropic properties. Various preparations or medications can also be purchased via the internet. The downside is the appearance of risk behaviours among some consumers (inability to drive, addiction or dependence) [8, 32]. The results of this pilot study indicate that kavalactones (kavain, 7,8-dihydrokavain, methysticin, 7,8-dihydromethysticin, 5,6-dehydrokavain (=desmethyoxyyangonin) and yangonin) accumulate in the keratin matrix of hair and can provide an acceptable and easily applicable system for assessing chronic consumption and abuse of kava. Because of numerous conjugated double bonds, kavalactones are easy to detect by HPLC-DAD. This preliminary study must continue on a larger number of subjects using GC/TOF-MS and LC-MS/MS in order to conduct a comparative analysis among the three methods.

Conflicts of interests. The authors have declared no conflicts of interests.

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