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Column-switching high-performance liquid chromatographic assay for determination of apigenin and acacetin in human urine with ultraviolet absorbance detection

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Abstract

A high-performance liquid chromatographic (HPLC) method is described for the determination of apigenin and the 4′-methylated derivative acacetin in human urine using column-switching and ultraviolet (UV) absorbance detection. Urine samples were enzymatically hydrolysed and solid-phase extracted prior to injection onto the HPLC system. Prior to elution of apigenin and the internal standard, 5,7,8-trihydroxyflavone, from the first column used for sample clean-up, the six-port valve was switched to the second column for analysis with UV detection. Detection of apigenin was precise and reproducible, with a limit of quantification of 10 ng ml^{-1} urine. Detection and quantification of acacetin was linear down to 70 ng ml^{-1} urine. The method has been successfully applied to determine the level of apigenin in 100 human urine samples from an intervention study with parsley.

Keywords: Apigenin; Acacetin

1. Introduction

Epidemiological and experimental studies suggests that a diet high in antioxidant flavonoids can reduce the risk for cardiovascular disease [1,2]. Apigenin is a flavone found in vegetables, seasonings and in oranges [3,4], and it possesses antioxidant activity in vitro [5,6]. Potent biological effects of this flavonoid have been described in vitro and in vivo. Apigenin has been described as anticarcinogenic, anti-inflammatory, and antimutagenic [7–10]. Thus development of a biomarker for exposure to apigenin is important in order to evaluate the potential health effects of this particular dietary component. There has only been one previous attempt to determine the urinary excretion of apigenin in humans after ingestion of an apigenin containing camomile extract [11]. However, due to lack of specificity and sensitivity, the method failed to detect any apigenin in the urine. Other HPLC methods have been developed for detection of various flavonoids in human urine samples, but none of these can readily be applied for detection of urinary apigenin. The method developed by Hollman and coworkers using post-column derivatization and fluorescence detection is limited to flavonols possessing a 3-OH group [12]. Since only a very small fraction of ingested flavonoids seems to be excreted in the urine of humans [13], a very sensitive method is required to detect the amount of apigenin excreted.

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We recently developed a sensitive HPLC method for detection of low levels of the flavonoid, quercetin, in human urine samples, using column switching and UV detection [14]. In this paper we report the development of a similar column switching HPLC method for the detection of low levels of apigenin in human urine samples using UV detection. In addition the method was able to detect the potential metabolite acacetin, the 4′-methylated derivative of apigenin. Methylation has previously been described in rodents as the primary metabolic pathway of flavonoids [15]. Enzymatic hydrolysis was used to liberate the urinary apigenin from glucuronic acid and/or sulphate conjugates. Parsley contain high amounts of apigenin, and it was therefore selected as the apigenin source for a human intervention study [16]. In this study the present method was successfully applied for the determination of the urinary level of apigenin.

2. Experimental

2.1. Chemicals and materials

Acetonitrile and methanol were of HPLC grade and obtained from Rathburne (Walkerburn, UK). Apigenin, acacetin (4′-methoxyapigenin), 5,7,8-trihydroxyflavone, apiin (apigenin-7-apiosylglycoside) and apigenin-7-glucoside were from Apin Chemicals (Oxon, UK), β-glucuronidase (*E. coli*, >200 standard units ml^{-1}) from Boehringer Mannheim (Mannheim, Germany) and arylsulfatase (*Aerobacter aerogenes*, 16.8 standard units ml^{-1}) from Sigma (St. Louis, MO, USA). All other chemicals used were of HPLC grade or reagent grade. Solid-phase extraction cartridges (Bond Elut, C-18, 500 mg) were purchased from Varian (Harbor City, CA, USA).

2.2. Instrumentation

The HPLC system consisted of a Hewlett–Packard (Waldbronn, Germany) 1090 system with three pumps, an automatic six-port column switching valve, and diode array detector. The columns used were a Lichrospher 60 RP-select B, RP-8 (125×4 mm, 5 μm) column with guard cartridge (4×4 mm, 5 μm) as column 1 and a Purospher RP-18 (125×4 mm, 5 μm) column with guard cartridge (4×4 mm, 5 μm) as column 2 (Hewlett–Packard). Column temperature was maintained constant at 40°C using a thermostatically controlled column compartment. Detection was carried out simultaneously at 290 and 350 nm, with peak scanning between 210 and 600 nm (2 nm step). The mobile phases used were (flow of 1 $ml\,min^{-1}$): A, 23.8 m*M* citric acid adjusted to pH 2.00 with conc. H_3PO_4; B, 100% acetonitrile and C: 100% methanol. A vacuum manifold from Waters (Milford, MA, USA) was used for the solid-phase extraction (SPE).

2.3. Standards

Apigenin and acacetin were dissolved in DMSO and 5,7,8-trihydroxyflavone (internal standard, see Fig. 1) was dissolved in MeOH (all 5 $mg\,ml^{-1}$) as stock solutions, and stored at −20°C. Spiked urine samples were prepared in quadruplicate by addition of the stock solutions of apigenin and acacetin to control urine samples, giving final concentrations of 0, 0.10, 0.50, 1.00, 2.50, 5.00, 10.00, 15.00, and 20.00 μg flavonoid per 15 ml of urine. 5,7,8-trihydroxyflavone was added as internal standard in an amount of 5 μg per 15 ml urine sample. Calibration curves were generated following enzymatic hydrol-

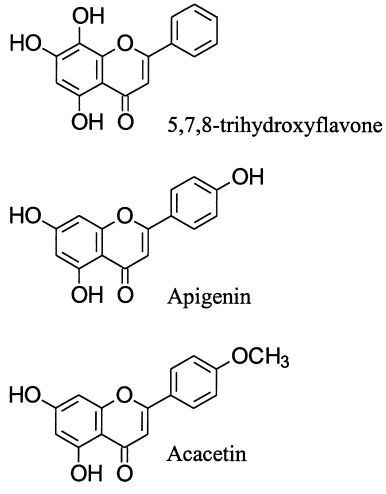

Fig. 1. Chemical structures of apigenin, acacetin and the internal standard, 5,7,8-trihydroxyflavone.

ysis, solid-phase extraction and HPLC analysis, as described in Section 2.5 Section 2.6 and Section 2.7.

2.4. Sample collection and preparation

As part of a human intervention study [16], 24 h urine samples were collected after consumption of parsley. The intake of apigenin with the parsley ranged from 33.5–87.8 mg day^{-1} (3.73–4.49 mg/MJ/day). The volunteers were on a controlled, energy balanced diet during the intervention period, low on flavonoids (the diet contained no fruits, wine or tea, or any vegetables known to contain flavonoids [17]). The vessels for urine collection contained 50 ml 1 *M* HCl and 10 ml 10% ascorbic acid as stabilisers and kept in the darkness during collection. The pH of the urine samples was adjusted to 3–4 by addition of approximately 50 ml 1 *M* HCl, and aliquots of 250 ml were stored at −20°C until analysed. Baseline urine samples were collected after seven days on controlled diet without parsley, and used as control urine.

2.5. Enzymatic hydrolysis of urine samples

An aliquot of 15 ml urine was bubbled with Ar (g) and adjusted to pH 5 with 2 *M* sodium acetate containing 10% ascorbic acid. As an internal standard, 5 μg 5,7,8-trihydroxyflavone was added to each sample. Enzymatic hydrolysis was performed by addition of 10 μl β-glucuronidase and 50 μl arylsulfatase preparation (>2 U and 0.7 U respectively) and the sample was incubated in a sealed vial for 1 h at 37°C under continuous shaking. After hydrolysis, the sample was applied to solid-phase extraction as described in Section 2.6.

The optimal incubation time for the enzymatic hydrolysis, was found by incubation of four different urine samples from the human intervention study in duplicate for either 15, 30, 60, 120 or 240 min at 37°C and subsequently performing solid-phase extraction and HPLC analysis.

Apigenin glycosides present in parsley [18] might be excreted unchanged in the urine after ingestion of parsley. The influence of the incubation with β-glucuronidase/arylsulfatase on these glycosides, was studied as follows. Apiin and apigenin-7-*O*-glucoside (2 μg each) were incubated in sodium acetate buffer for 1 h in triplicate, with or without the enzyme preparation under the same conditions as described above, but in a total volume of 2 ml (10 μl β-glucuronidase and 50 μl arylsulfatase used). After incubation, 100 μl of each sample was analysed by HPLC using the isocratic elution mode on column 1, in comparison with a 100 μl aliquot taken prior to the incubation.

2.6. Solid-phase extraction

Solid-phase extraction was performed as described previously [14]. Briefly, after preconditioning of a Bond Elut C-18 cartridge (500 mg), the hydrolysed urine sample was applied to the column with a flow of approximately 5 ml min^{-1}. The cartridge was washed with 3 ml 5% methanol, 1% formic acid and eluted with 3 ml 90% methanol (1% formic acid, 1% ascorbic acid) followed by 3 ml pure methanol. The eluates were combined and evaporated to dryness under vacuum. The residue was dissolved in 250 μl 20% acetonitrile, 1% formic acid and 100 μl was injected onto the HPLC system.

2.7. HPLC conditions for analysis of urine samples

A schematic diagram of the HPLC system is shown in Fig. 2. The sample was injected onto column 1 with the column-switching valve in position 1, using isocratic elution from 0–32 min, with 20% acetonitrile (B) in mobile phase A (v/v). Between 19–28 min, the switching valve was shifted from position 1 to 2, thus eluting the target compounds 5,7,8-trihydroxyflavone and apigenin onto the second column. Between 28 and 44 min the switching valve was shifted to position 1. From 32–39 min column 1 was eluted with a linear gradient from 20%–100% B in A (v/v), whereas acacetin was eluted from column 1 through the detector. Between 39 and 40 min column 1 was washed with pure acetonitrile (B) with the switching valve in position 1. After preconditioning of column 1 from 40.1–44 min with 100% citric acid buffer (A), the switching valve was shifted from position 1 to 2 at 44 min. The flavonoids were eluted from column 2, using a gradient (v/v) of mobile phase A and methanol (C): 44 min: 0% C, 50 min: 45% C, 60

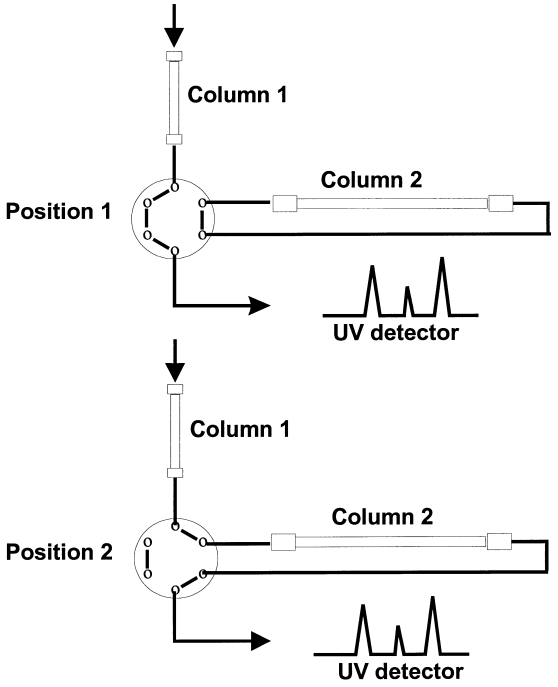

Fig. 2. Schematic diagram of the column switching HPLC system.

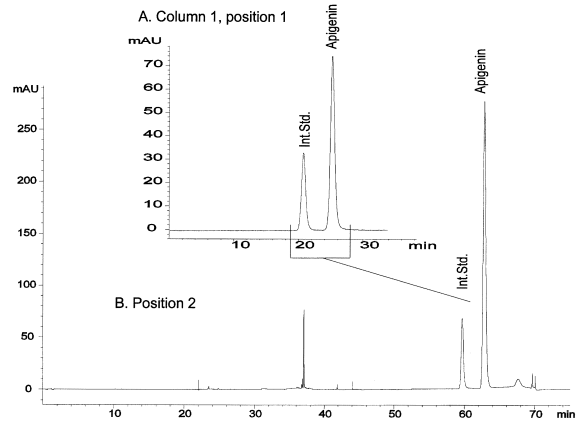

Fig. 3. Chromatograms showing (A) the elution of the internal standard, 5,7,8-trihydroxyflavone and of apigenin on column 1, using isocratic elution. Time-points of column switching indicated with frames. (B) The internal standard and apigenin after elution from column 2, detected at 350 nm.

min: 50% C, 64 min: 60% C, 65–67 min: 100% C, 67.1–75 min: 100% A.

2.8. Reproducibility and stability

The reproducibility and stability of the assay was ensured by analyses of external standards before and after each series of samples as described previously [14]. As external standard, 15 μl of a mixture of 0.2 μg μl^{-1} 5,7,8-trihydroxyflavone and apigenin in methanol was injected onto column 1 with isocratic elution, (20% acetonitrile (B) in mobile phase (A) and the column-switching valve in position 1, ensuring that the target compounds elute within the time-frames of column-switching (see Fig. 3). Furthermore, the reproducibility of the complete HPLC method was controlled by injecting another 15 μl of the standard mixture. When setting up large series of samples, one urine sample was selected as control sample (No. 1091-04) and included repeatedly with each series of samples to ensure reproducibility of the method.

The stability of the internal standard, 5,7,8-trihydroxyflavone and apigenin during the enzymatic hydrolysis was investigated by incubating 10 μg of 5,7,8-trihydroxyflavone and apigenin respectively, in 1 ml acetatebuffer at pH 5 or at pH 6. The samples were prepared in triplicate and analysed by HPLC prior to and after the incubation for 1 h at 37°C under continuos shaking.

The stability of apigenin and acacetin in human urine, was assessed by spiking control urine samples with 2500 ng of each flavone per 15 ml sample. The samples were kept at −20°C until analysed. Prior to analysis 5 μg 5,7,8-trihydroxyflavone was added as internal standard.

3. Results and discussion

3.1. High-performance liquid chromatography of urine samples

To obtain sufficient separation of apigenin from interfering compounds in the urine, it was found necessary to use a column-switching method, and to apply different conditions to the two columns. Based on previous investigations of various stationary phases for flavonoid detection, using column-switch-

ing, a Lichrospher RP-select B, RP-8 (125×4 mm) column was selected as the first column, and a Purospher RP-18 (125×4 mm) column as the second column [14]. By the application of acetonitrile as the organic solvent on column 1 and of methanol as the organic solvent on column two, sufficient separation of the target compounds, apigenin and the internal standard (5,7,8-trihydroxyflavone) from interfering compounds in urine was achieved. Furthermore, the application of isocratic elution on the first column, gave a very selective elution of the target compounds. Before the elution of 5,7,8-trihydroxyflavone and apigenin, from column 1, the automatic six-port valve was programmed to switch from position 1 to position 2, selectively eluting the two compounds onto column 2. After the elution of the target compounds onto column 2, the more unpolar impurities including the methylated derivative of apigenin, acacetin, remaining on column 1, were eluted by a quick column wash with 100% acetonitrile, followed by reequilibration with 100% citric acid buffer, with the six-port switching valve in position 1. Finally a gradient of methanol and citric acid buffer was applied to column 2, eluting the compounds through the UV detector. Typical chromatograms of a blank urine, a spiked urine sample, and a urine sample obtained after parsley intervention are shown in Fig. 4. The internal standard, 5,7,8-trihydroxyflavone, and apigenin were highly separated with retention times of 59.1 and 62.2 min respectively. As seen in the chromatograms obtained at 350 nm (Fig. 4A, B, C) practically no interfering peaks were observed where apigenin and 5,7,8-trihydroxyflavone elutes, whereas more interfering compounds were seen where acacetin (36.8 min) elutes, giving a higher detection limit for the latter compound. Comparison of the UV-spectrum of apigenin detected in a urine sample, shows good similarity with authentic apigenin (see insert in Fig. 4) and enables positive identification by comparison of the UV-spectra. Reproducibility of the retention times of the target compounds on column 1 was crucial for the method, since variations could result in one of the compounds eluting outside the time-frames of column switching. Thus it was necessary to control the retention times of 5,7,8-trihydroxyflavone, and apigenin on column 1 (see Fig. 3), prior to and after setting up a series of samples, with the switching valve in position 1 using isocratic elution with 20% acetonitrile in citric acid buffer.

3.2. Enzymatic hydrolysis

The enzymatic preparation from *Helix pomatia* previously used for the hydrolysis of the glucuronic acid and sulphate conjugates of flavonoids found in urine [14], was initially assessed for this method. However, to our surprise, this enzyme preparation was found to be contaminated with small amounts of apigenin, why this preparation was not suitable for the present assay (see Fig. 5.). Therefore a β-glucuronidase from *E. coli* and an arylsulfatase from *Aerobacter aerogenes* was selected for this study instead.

The optimal conditions for the enzymatic hydrolysis of the urine samples were determined to be 1 h of incubation (see Table 1).

The stability of 5,7,8-trihydroxyflavone and apigenin during the enzymatic hydrolysis was investigated at different pH. After 1 h of incubation at pH 5 >90% of both 5,7,8-trihydroxyflavone and apigenin was recovered, but at pH 6 >85% of 5,7,8-trihydroxyflavone, and only >45% of apigenin was recovered.

We previously reported that the enzyme preparation from *Helix pomatia* is able to hydrolyse quercetin glycosides [14]. However, the mixture of β-glucuronidase and arylsulfatase used in the present assay was not able to hydrolyse the apigenin glycosides present in parsley, apiin and apigenin-7-glucoside (data not shown). Thus to determine whether apigenin glucosides were excreted unchanged into the urine, the urine sample with the highest content of apigenin from the intervention study (No. 1366-03) was analysed after enzymatic hydrolysis by LC–MS using the method described by Nielsen et al. [19] in comparison with authentic standards of apiin, apigenin-7-glucoside and apigenin. The presence of the molecular ion at m/z 269 $[M-1]^-$ verified the identification of apigenin in the urine sample, but no apiin, apigenin-7-glucoside or acacetin were detected in the extracted ion tracks at their respective molecular ions (data not shown, detection limit determined

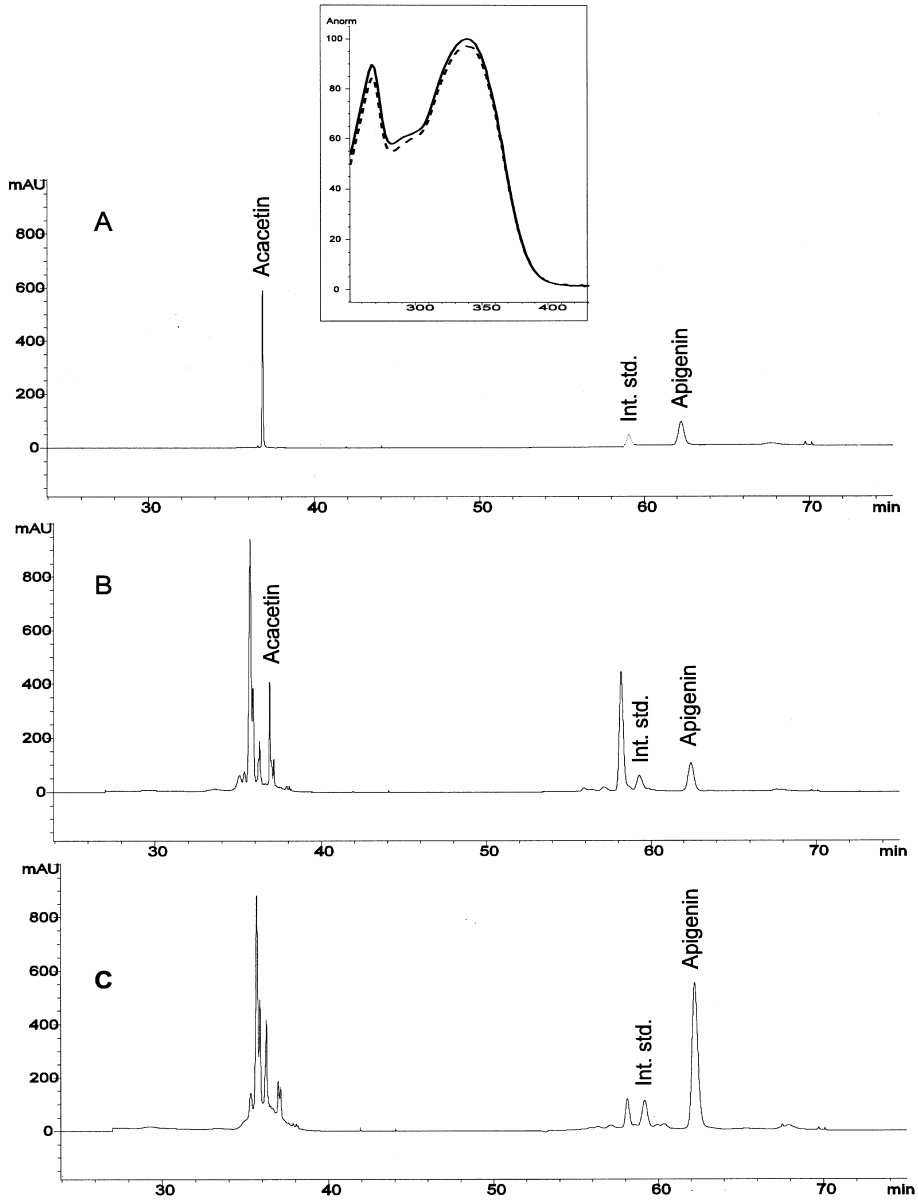

Fig. 4. Chromatograms at 350 nm of (A) 1 μg each of acacetin, 5,7,8-trihydroxyflavone (Int. Std.) and apigenin, (B) control urine spiked with 1 μg acacetin, 5,7,8-trihydroxyflavone (Int. Std.) and apigenin, (C) a urine sample collected during intervention study with parsley spiked with 5 μg int. std. UV-spectra recorded online with diode array-detector of the apigenin peak in urine (solid line) compared with authentic apigenin (dotted line) are shown as insert.

as $S/N>3$: 5 ng ml^{-1} urine). Thus apigenin is apparently only present as glucuronic acid or sulphate conjugates in the urine, and none of the glycosides were found to be excreted as such in the urine in detectable quantities.

3.3. Linearity, detection limits, reproducibility and stability

Calibration curves were obtained by enzymatic hydrolysis, SPE and HPLC analysis of spiked control

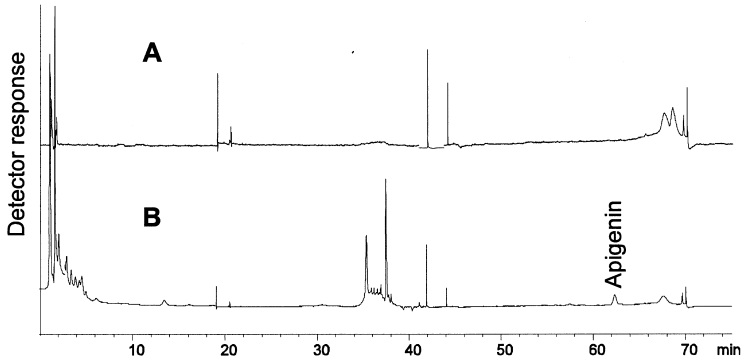

Fig. 5. Chromatograms of the enzyme preparation used in this assay for enzymatic hydrolysis of the urine samples (A) compared to the enzyme preparation used previously from *Helix pomatia* [14].

urine samples over the concentration range 0.10–20.00 μg per 15 ml urine. The equally weighted correlation between peak area determined at 350 nm and the concentration of apigenin was linear between 0.10–20.00 μg per 15 ml urine, passing through the origin, with a correlation coefficient better than 0.999. The limit of quantification, as determined by spiking of control urine was 10 ng ml^{-1} urine for apigenin at 350 nm. The correlation between peak area (at 350 nm) and the concentration of acacetin was linear between 1.00–20.00 μg per 15 ml urine, with a correlation coefficient of 0.998 and a limit of quantification, as determined by spiking of control urine at 70 ng ml^{-1} urine. The reproducibility of the HPLC method was assessed by the external standard mixture of 0.2 μg μl^{-1} of 5,7,8-trihydroxyflavone and apigenin analysed prior to and after each series of samples. The retention times of the external standard showed an inter- and intra-day variation of less than 1% (coefficient of variation, C.V.%) and the peak areas determined varied less than 5% (C.V.%, $n=20$). Apigenin and acacetin added to urine samples (2500 ng per 15 ml sample) was found to be stable (defined as ≥95% of initial amount remaining) for at least 12 months when stored at −20°C.

3.4. Application of the method

The present method was applied for the analysis of more than 100 urine samples collected from 14 individuals over a 14-day intervention period. Details will be published elsewhere [16]. Apigenin was detected in all the urine samples collected after intervention with parsley and the levels in control samples were below the detection limit. The urinary apigenin excretion in 24 h was 657.36±385.98 mg (mean±SEM, $n=14$, range: 24.44–5727.32 mg) after one week of parsley intervention. This corresponds to an average excretion of 0.58%±0.16 (± SEM) of the apigenin dose in 24 h. One of the urine samples (No. 1091-04) kept at −20°C, was included as control sample in each series of analyses over a period of three months. The amount of apigenin detected in this sample was 550±18.1 ng ml^{-1} (mean±S.D., $n=10$). The amount of apigenin excreted in urine does not necessarily reflect the

Table 1
Optimisation of the conditions for the enzymatic hydrolysis of urine samples

	Incubation time (min) for enzymatic hydrolysis				
	15	30	60	120	240
Amount of apigenin detected[a]	88.0%±5.6	94.7%±2.3	100%	96.8%±2.7	89.2%±2.5

[a]The amount was determined as percentage of the maximum amount of apigenin detected by HPLC analysis (at 60 min).

absorbed amount of apigenin, since biliary excretion is also a possibility. However, recent investigations failed to detect any apigenin in blood samples of subjects after intake of dried parsley [20], and therefore, urinary apigenin might be a useful marker of dietary exposure.

The recovery of the internal standard, 5,7,8-trihydroxyflavone, was higher than 90% in all samples. Since similar recoveries of apigenin and of 5,7,8-trihydroxyflavone were found in spiking experiments, the detected amount of apigenin in each of the analysed urine samples was individually corrected for analytical loss of internal standard.

The usefulness of this method for analysis of blood samples were not evaluated. However, the methodology of column-switching may be applied in general for simultaneous determination of 2–4 different flavonoids. Larger numbers of flavonoids will allow too many impurities to be transferred to column 2, thereby impeding the separation of compounds of interest. Increasing the numbers of compounds for simultaneous determination, and thus increasing the time frames of column switching may also lead to peak broadening of the compounds eluted onto column 2.

4. Conclusions

The method described in this paper represents a specific and sensitive assay for the determination of apigenin and the potential 4′-*O*-methylated metabolite, acacetin, in human urine samples. To our knowledge, this is the first method described for the determination of apigenin and acacetin in human urine. The method was successfully applied to a human intervention study [16]. The use of SPE and column-switching in the present assay gave a 100-fold concentration of the urine samples, enabling the detection of low levels of urinary apigenin. This method would therefore be suitable to determine apigenin in human urine as a marker for the dietary exposure to this specific flavone.

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