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Chapter 7

Microfractionation Revisited: A 1536 Well High Resolution Screening Assay

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Abstract

The aim of the here presented study was to combine high performance liquid chromatography with plate reader technology in order to overcome certain drawbacks of integrated online systems as well as offline plate reader approaches. The described method combines an “at-line” enzyme assay for the simultaneous bioactivity determination with parallel QTOF MS data acquisition for analyte identification. All biochemical reagents are added in an online mode directly to the column effluent (postcolumn addition/mixing), and the complete screening assay mixture is subsequently microfractionated into a 1536 well plate. The screening of a natural extract fortified with two well-known Protein Kinase A inhibitors and the identification of an inhibitor in a natural extract showed the applicability of the approach to detect bioactive compounds in low concentrations in a complex mixture. The described mode of operation utilizes today’s plate reader technology to its full capacity and directly hyphenates it to a high resolution separation technique which has not been shown before. Furthermore, it allows coupling of a microbore HPLC with a biochemical screening assay without compromising resolution and overcomes problems associated with the 1536 well format.

Introduction

In today’s drug discovery environment complex mixtures such as natural extracts [1, 2], fragment based libraries, or combinatorial approaches become more and more important for lead structure finding [3, 4]. The complexity of such substance mixtures demands an initial substance separation to provide a fast and reliable inhibitor/effector detection and identification. High-performance liquid chromatography (HPLC) in combination with fractionation techniques is extensively employed to unambiguously identify bioactive compounds. Using low resolution, offline approaches fractions are typically collected in tubes or 96 well plates [5, 6], and subsequently the biochemical reaction-detection of possible enzyme inhibitors/effectors is performed by plate reader assays. Alternatives to this approach are online high resolution screening systems (HRS) [7] which combine analyte separation and biochemical detection in an integrated system. Both approaches have drawbacks in either resolving highly complex mixtures with an efficient logistics or performing biochemical assays with long incubation times. Plate reader assays have the benefit that several different read-out technologies can be applied easily by using a single system. But in order to maintain chromatographic resolution, the commonly used 96- or 384-well formats are less beneficial. Problems with the 1536 well format that would allow high-resolution fractionation are basically related to liquid handling which is normally done by pipetting robots [8], acoustic liquid handling devices, or other technologies. These devices can have the disadvantage of high dead volumes which is particularly relevant when handling very expensive and rare enzymes or chemicals. Moreover, foam formation can severely influence the assay performance. Finally, an efficient mixing within the wells of a 1536 plate is only possible by

resuspending the whole well content because of the high surface to volume ratio [9], hence homogeneous premixed assays are best suited for application in miniaturized 1536 well formats [10].

The aim of the present work was to combine the benefits of online and offline screening methodologies and to develop a 1536 well microfractionation technique for bioactivity guided screening purposes which allows for a very fast fraction collection while maintaining the high resolution achieved by the LC separation. This also allowed hyphenating a high resolution microbore LC separation, to a biochemical detection reaction.

Until now there are only a few microfractionation systems described collecting at fraction times in the minute(s) range; [6, 11, 12] the shortest described fraction time was 0.3 min [5, 13]. In the proteomics field a nanofractionation system was described by Corso et al., [14] with fractionation volumes in the order of nanoliters and fractionation times in the range of 15–120 s. These relatively long sampling times, however, do not maintain the resolution achieved by the LC column, especially when using microbore columns. The biological testing of the above-mentioned microfractionation based screening methodologies was mainly done after fraction evaporation. This again gives rise to mixing problems and other issues discussed above, especially when working in a highly miniaturized 1536 well format. Therefore, we developed an online addition of all biochemical reagents necessary for the biological read out prior to the microfractionation step. In this way a 1536 well format can be employed while avoiding mixing issues, plate drying, resolvation, and multiple pipetting steps but still allowing long incubation times without affecting the resolution of the enzymatic assay and using the full capabilities of today's plate reader technology.

For the development of the described assay methodology we choose a protein kinase (PKA - protein kinase A), because many enzymes of the kinase family are expensive and rare, so there is a true need of miniaturization. The readout technologies for kinase assays are mainly based on fluorescence polarization (FP) employing immobilized metal ion affinity (IMAP)-based fluorescence polarization beads, time-resolved fluorescence resonance energy transfer (FRET), or luminescence [10]. The luminescence readout methodology is a robust and reliable technique employing luciferase and luciferin, thereby determining the residual ATP in the reaction mixture [15]. Due to the high sensitivity and the relatively simple principle we employed the luminescence readout for the development of the LC microfraction-biochemical assay methodology.

Experimental Section

Chemicals

Kinase-Glo reagents and PKI 5-24 were purchased from Promega (Leiden, The Netherlands). Malantide was purchased from Bachem (Bubendorf, Switzerland). The Isolute C18 SPE columns 500 mg, 6 mL were from Biotage (Uppsala, Sweden), and the *Cistus incanus* extract (3:1, 20% ethanol) was from Gehrlicher herbal extracts (Eurasburg, Germany). All other chemicals were purchased from Sigma Aldrich (Schnelldorf, Germany).

Instrumentation

We used a Shimadzu (Duisburg, Germany) LC20 AB quaternary pump to deliver a mixture of water acetonitrile (0.05% formic acid) at 60 $\mu\text{L}/\text{min}$ (effluent A water/ACN 90:10, effluent B ACN/water 90:10). The autosampler was a SIL 20 A (Shimadzu, Duisburg, Germany). The column was an XTerra MS C_{18} 3.5 μm , 1 \times 150 mm (Waters, Milford, MA, USA). The mass spectrometer (MS) was a micromass Ultima Q-TOF (Waters, Milford, MA, USA). The MS was operated in ESI+ mode, if not otherwise stated, the capillary voltage was 3 kV, the source temperature was 120 $^{\circ}\text{C}$, the desolvation temperature was 350 $^{\circ}\text{C}$, and the cone voltage was 35 V. Nitrogen (99.9990%) was used at flow rates of 50 L/h as cone gas and 400 L/h for desolvation. The MS scanned from m/z 300 to 800; in the case of the *Cistus* extract the MS scanned from m/z 220 to 850. The positive ESI MS/MS experiments were done under the same conditions, with a collision energy of 30 V, an isolated ion with m/z 465, and a scan range of m/z 100–500, the negative ESI MS/MS measurements were done under the same conditions at -3 kV, and the isolated ion was m/z 463. Substrate and enzyme solution were pumped by a two channel syringe pump model 22 (Harvard apparatus, South Natick, MA, USA). The Model 234 autosampler serving as fractionator was from Gilson (Middleton, USA), and the needle of the “autosampler” was replaced by a 150 μm i.d. deactivated fused silica capillary, of which the last cm of the polyimide coating was removed and the residual quartz glass was silylated (Supplementary material S3 shows a picture of the silylated glass capillary) using dimethyldichlorosilane in toluene 5% for 3 min. The software controlling the Gilson 234 was homemade. 1536 well plates, solid, white, 10 μL were from Brand (Wertheim, Germany). 96 well plates, solid, white were from Greiner Biosciences (Solingen, Germany). The plate readers employed were a Victor(3) and a Victor(2) (Perkin-Elmer, Waltham, MA, USA). The Victor(3) counted chemiluminescence for 0.5 s per well. The Victor (2) counted 1 s per well (96 well experiments).

PKA Assay, IC_{50} Determination, and Screening Procedure

For IC_{50} determination the system was operated under isocratic conditions at 60% of effluent B with the exception for staurosporine where a mobile phase containing 75% of effluent B was employed. For the analysis of mixtures a linear gradient was used starting at 0% B, hold for 0.5 min, to 85% B at 19 min, hold for 2 min (in the case of the *Cistus* extract, hold for 6 min). For all experiments injection volumes of 5 μL were used. The column effluent was split 1:9 into the enzyme assay (6 $\mu\text{L}/\text{min}$ to the biochemical assay, 54 μL to the MS). The two channel syringe pump supplied enzyme solution (PKA 0.125 U/ μL in TRIS HCl buffer 40 mM pH 7.5, MgCl_2 20 mM, 0.2 g/L BSA) and substrate solution consisting of malantide 25 μM , ATP 12.5 μM , cAMP 50 μM , MgCl_2 20 mM, PEG 6000 2 g/L, and glycerol 20% (m/m) in TRIS HCl buffer 40 mM pH 7.5 at flow rates of 22 $\mu\text{L}/\text{min}$. The plates were incubated for a total time of 30 min. The Gilson 234 was programmed to collect fractions of 2.5 s, if not otherwise stated. The kinase Glo assay was diluted 1:1 with TRIS buffer and subsequently supplied to the wells using a second Gilson 234 at a flow rate of 60 $\mu\text{L}/\text{min}$ and a fraction time of 2.5 s (keeping the same time frame of the microfractionation). After addition of the Glo assay reagent, plates were read at the Victor(3) plate reader after an additional wait time

of 5 min. For the fortified extract experiments we used a combination of a *Lupuli strobulus* (5.5–6.5:1; water) and *radix valerianae* (5.3–6.6:1; 45% MeOH) extract (1:4) at a final concentration of 5 mg/mL in water/ACN 95:5, 0.05% HCOOH, spiked with the PKA inhibitors staurosporine (4 μ M) and PKI 5-24 (10 μ M), and the whole chromatogram was fractionated. In the case of the *Cistus incanus* extract, 40 mg of the extract was weighed into a 2 mL plastic tube and suspended in 2 mL of 10% MeOH. The tube was vigorously shaken for 5 min and centrifuged at $10.000 \times g$ for 3 min. The upper solution was transferred onto a 500 mg C18 SPE cartridge, which was conditioned with 3 mL of MeOH and water each. The cartridge was washed with 4 mL of water, dried under full vacuum, and eluted with 3 mL of MeOH. The MeOH eluent was evaporated to dryness under a gentle stream of nitrogen at room temperature. The residue was resolved in 1 mL of 10% ACN, 0.1% formic acid and centrifuged again for 3 min at $10.000 \times g$. The solution was either directly injected into the system (5 μ L) or 200 μ L was spiked with 50 μ L of an aqueous solution of PKI 6-22 (100 μ M) and 7 μ L was injected. All calculations were done with Graph Pad Prism 4.

96 Well Experiments

96 Well Fractionation

The column effluent was directly connected to the Gilson 234, without splitting. The Gilson was programmed to collect 20 s fractions. The separation of the inhibitors was accomplished as described for the 1536 well fractionation. Fractions were collected from 10 to 20 min. The so prepared plate was dried under vacuum (about 100 mbar) for 1.5 h. Twenty μ L of each substrate and enzyme solution were added, and the plate was gently shaken for 60 s. The plate was further incubated for 29 min. 50 μ L of the diluted Glo assay reagent was added, and after an additional incubation of 5 min the plate was read by a Victor(2) plate reader.

Influence of the Acetonitrile Concentration

To each well of a 96 well plate were added 22 μ L of substrate solution (see above) followed by varying amounts of water 0.05% HCOOH and ACN 0.05% HCOOH, to reach the final ACN concentration. After gentle mixing 2 μ L of PKI 5-24 stock solution in TRIS buffer (5 μ M) and 22 μ L of enzyme solution were added. The plate was gently shaken for 60 s and treated further as described above.

Results and Discussion

The developed system (Fig. 1) consists of a gradient reversed phase HPLC separation using a microbore column (1 mm) coupled online to a quadrupole-time-of-flight mass spectrometer (QTOF MS) for real time high resolution mass spectra recording, a five port mixing device (one port closed) for the addition of enzyme (PKA), cofactors (Mg^{2+} , cAMP), and substrate (ATP), coupled to a Gilson 234 autosampler, serving as microfractionator (sample processing robot) (pictures of the described setup are shown in the Supplementary material S1-S3, S6 provides a video of the operating system. After substance separation the column effluent is split 1:9. One part is directed toward the biochemical assay, and nine parts are directed toward

the QTOF MS. Before fractionation is accomplished by the Gilson 234 all necessary biochemicals are added online to the effluent stream via syringe pumps. The software controlling the Gilson 234 was written by us. The employed PKA assay conditions have been optimized and described by us elsewhere; [16] if not investigated during the cited study, or different in the present study, the conditions were optimized in a 96 well plate. To characterize the described methodology we investigated the z' -factor [17], the IC_{50} values of the PKA inhibitors staurosporine [18], PKI 6-22 and PKI 5-24 [19], intraday repeatability, and interday repeatability as well as extract fortification experiments. The inhibitor experiments were carried out at about 90% substrate carry over under the below stated conditions. Repeatability experiments were done with pure standard solutions of the inhibitors. The IC_{50} values were determined under isocratic conditions, taking care that elution took place in the dead time of the column.

Comparison with 96 Well Fractionation

Fractionation is usually done by collecting the column effluent in glass tubes or 96 well plates. To give a direct comparison between the 1536 well microfractionation format and a traditionally used 96 well format we connected the LC column directly to the fractionation capillary of the Gilson 234. Figure 2 clearly shows the superior resolution of the 1536 well assay, thereby allowing for a real peak shape comparison between liquid chromatography and biochemical detection. This is especially useful when highly complex mixtures are tested [20]. Figure 3 shows an additional, virtual comparison of different fractionation times. The virtual comparison was done by averaging the time points from a 2.5 s fractionation, resulting in virtual fractionation times of 5 s (2 points averaged), 10 s (4 points), and 20 s (8 points).

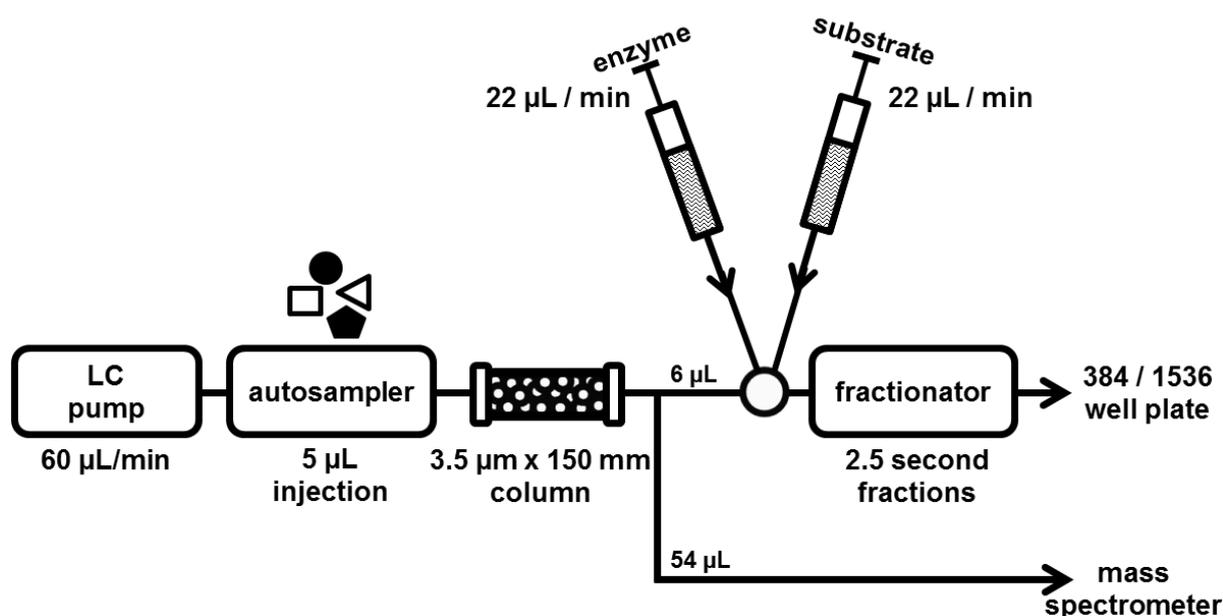


Figure 1. Schematic overview. For details refer to the Experimental Section.

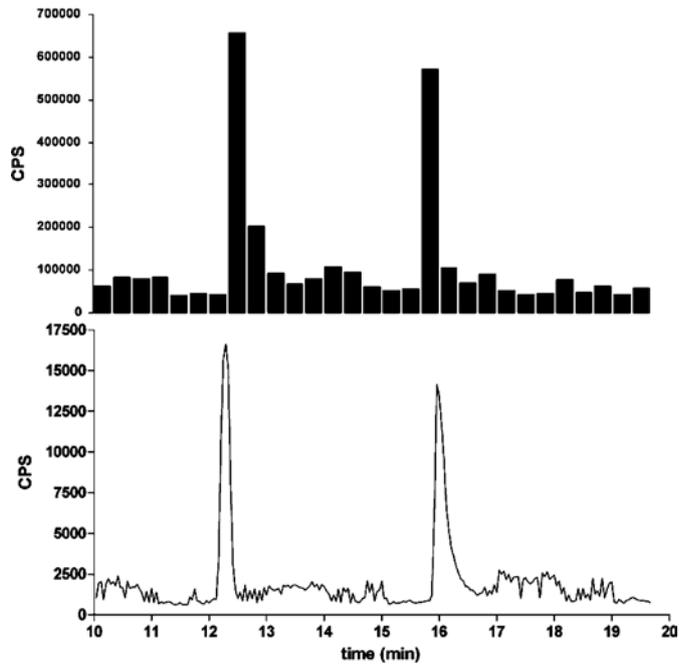


Figure 2. Direct comparison between 96 and 1536 well fractionation. Above 96 well plate - 20 s. fractions, below 1536 well plate - 2.5 s fractions. CPS counts per second.

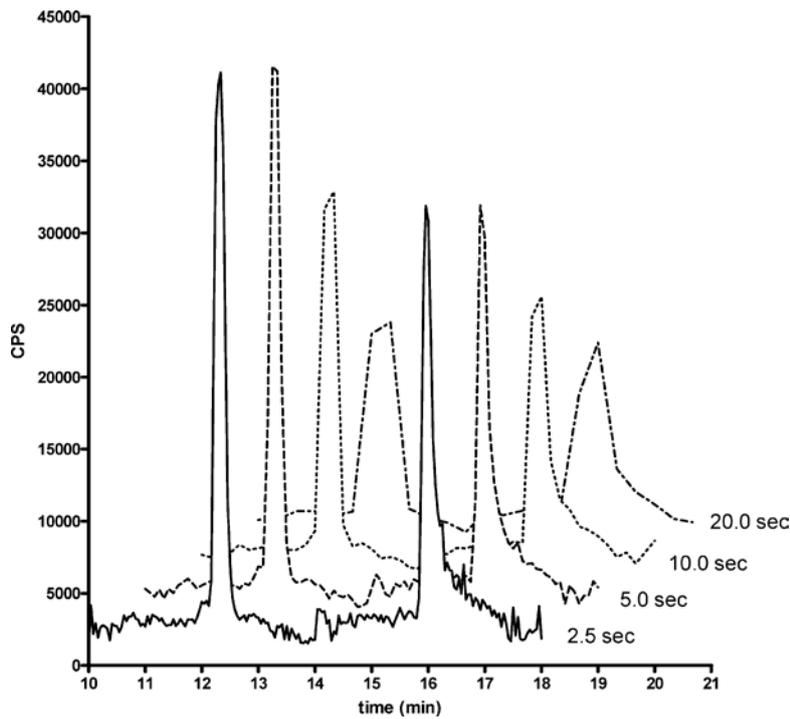


Figure 3. Virtual comparison of different fraction times. 2.5, 5.0, 10, and 20 s, data were collected at a fractionation time of 2.5 s. 5.0 s is equal to the average of two data points, etc. CPS counts per second.

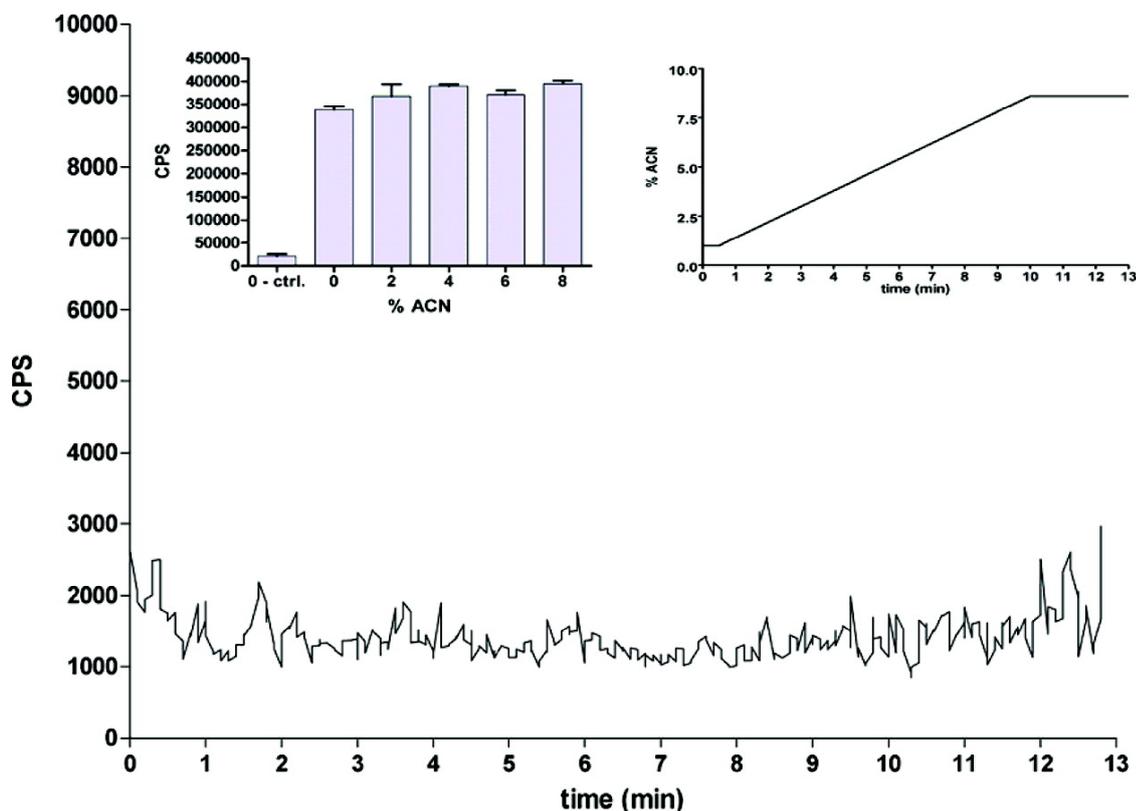


Figure 4. Influence of acetonitrile content. ACN acetonitrile, CPS counts per second, 0 - ctrl. control experiment without inhibitor addition at 0% ACN, above left inhibitor response of 200 nM PKI 5-24 at different ACN concentrations, error bars show one SEM, above right course of the applied gradient, ACN content in the reaction buffer, below biochemical trace running an ACN gradient from 1 to about 8%.

Influence of Acetonitrile Content on the Biochemical Reaction Detection

To investigate the influence of different acetonitrile contents on the biochemical reaction we investigated the baseline (no inhibitors present) of a fractionated acetonitrile gradient. Furthermore the signal intensity of PKI 5-24 was investigated at 0, 2, 4, 6, and 8% ACN in the reaction buffer in a 96 well plate. Applying a one-way ANOVA test ($P < 0.05$) there was no significant difference between the inhibitor responses at the different ACN concentrations. The baseline of the described experiment was stable up to an ACN content of about 8% (4% for luciferase reaction) as shown in Figure 4. Hence, when performing a gradient elution in screening mode, this stability allows for an ACN concentration of up to 85% ACN, as only 10% of the eluent is used for biochemical detection.

Extract Fortification Experiments and the Detection of Myricetin-3-O-rhamnoside

To show the ability of the current method to detect inhibitors in complex mixtures we fortified a combination of a *valerianae radix* and *lupuli strobulus* (4:1) extract with the PKA inhibitors staurosporine (4 μM) and PKI 5-24 (10 μM) (Fig. 5). It can be seen that the enzyme activity chromatogram clearly shows the two active compounds which can only be found in the extracted ion mode or the base peak intensity mode. Again, in this situation a high resolution

fractionation, as accomplished in the 1536 well format, allows to “pick” a single peak of the HPLC separation, resulting in a high resolution MS trace which is associated with the biochemical activity. This allows identifying known substances by database comparison (i.e., www.massbank.jp [21]), receiving the sum formula of unknown compounds and using a bioactivity directed isolation procedure of unknown substances. A proof for these statements could be given by the identification of myricetin-3-O-rhamnoside as a PKA inhibitor in a *Cistus incanus* extract. During the screening of different extracts we could detect an inhibitory effect of a *Cistus incanus* extract. The microfractionation of the SPE cleaned extract revealed a peak with m/z 465 to be at least partially responsible for the effect (Fig. 6). The ESI+ MS/MS spectra of the substance showed a very good matching with the spectra published in the massbank library (www.massbank.jp). The identity was additionally confirmed by the comparison of the ESI- MS/MS data with literature; [22] overall the obtained MS and MS/MS data allow the conclusion that the found substance is with a very high probability myricetin-3-O-rhamnoside (for the MS/MS spectra see Supplementary material S4). The experiment was repeated with the additional fortification of the extract with PKI 6-22 to allow a very precise time correlation between separation and activity trace. Furthermore the finding of myricetin-3-O-rhamnoside stands in good accordance with the well-known PKA inhibitory effect of myricetin itself [23] ($K_i = 27 \mu\text{M}$).

Repeatability and z' -Factor

Intraday repeatabilities for a standard solution of staurosporine ($4 \mu\text{M}$) and PKI 5-24 ($10 \mu\text{M}$) were 8.1 and 13.7% (coefficient of variation, CV, for $n=3$), respectively; interday repeatabilities were 31.6% and 31.2% (CV for $n=6$), respectively. These are fairly good results which are acceptable for a screening technology. The z' -factor, calculated according to Zhang et al. [17], was 0.75 determined by comparing the signals with and without enzyme ($n = 140$), signal-to-background was 63, and signal-to-noise was 166. A z' -factor of 0.75 demonstrates the excellence of the developed technology, as assays having a z' -factor of higher than 0.5 are considered to be very good [17].

IC_{50} and K_i Values

To demonstrate the comparability with other assays and to establish a clear concentration to signal dependency we determined the IC_{50} values for PKI 5-24, PKI 6-22, and staurosporine. As shown in Table 3 and Figure 7 the resulting IC_{50} curves exhibit excellent R^2 values. The resulting IC_{50} and K_i values are higher than the values reported in the literature. This can basically be explained by the longer incubation time, the higher amount of enzyme used in this study (factor 2 compared to ref 16), and a different read out as compared to the cited studies. K_i values were calculated using the equation $IC_{50} = K_i(1+[S]/K_m)^{24}$, with $[S] = 11 \mu\text{mol/L}$ malantide and K_m (malantide) = $15 \mu\text{mol/L}$ [25, 26]. The obtained IC_{50} values were corrected by a dilution factor of 76.6, which was determined as described elsewhere [27]. For a brief description see Supplementary material. Figure 8 shows the recorded biochemical trace of the IC_{50} determination of PKI 6-22.

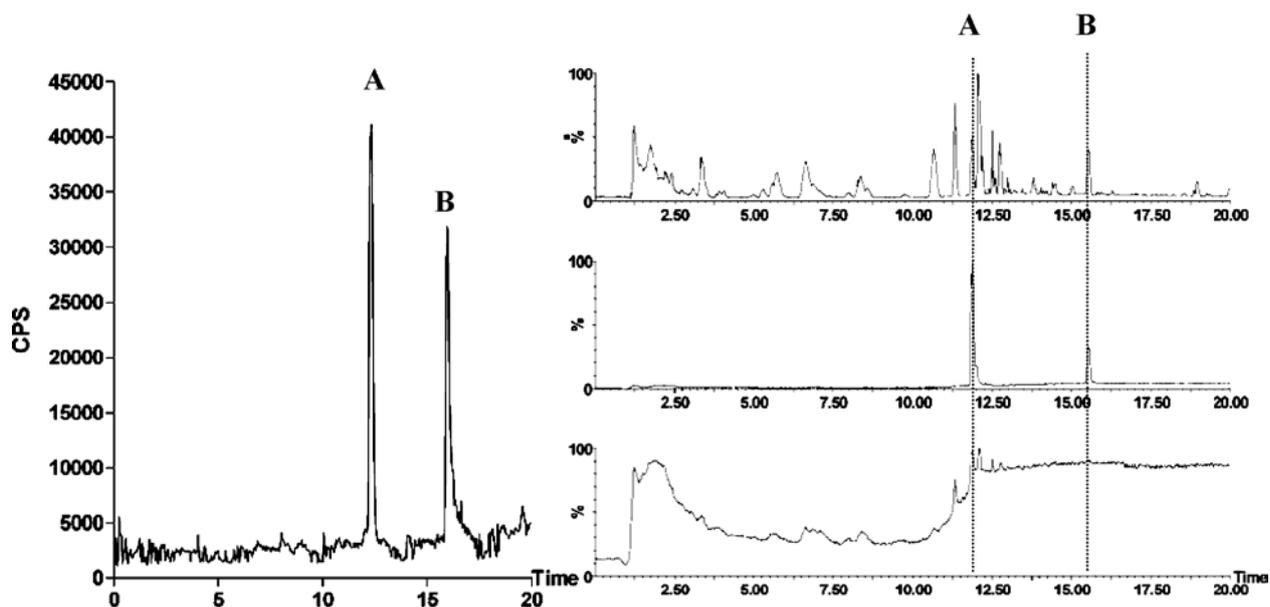


Figure 5. Fortified plant extract - 10 μM PKI 5-24 (A) and 4 μM staurosporine (B). CPS counts per second, left enzyme activity trace, right top BPI (base peak intensity) chromatogram, right middle EIC (extracted ion chromatogram) m/z 445, 556, 467, right bottom TIC (total ion chromatogram).

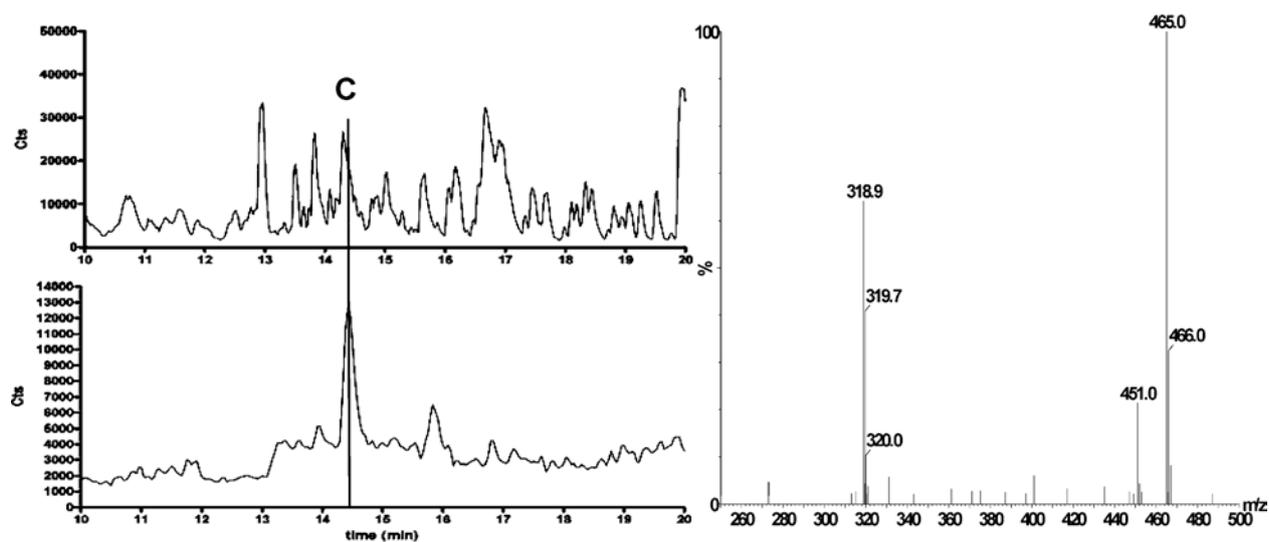


Figure 6. Detection of myricetin-3-O-rhamnoside in *Cistus incanus*. Above left BPI chromatogram of the *Cistus incanus* extract, below left activity trace, C myricetin-3-O-rhamnoside, right MS spectrum of C.

Table 3.
IC₅₀ and K_i Values^a

substance	IC ₅₀ (nM)	K _i (nmol/L)	R ²	ref K _i (nmol/L) [18, 19, 28]	ref IC ₅₀ (nM) [16, 18]
PKI 5-24	48	28	0.9944	2.3	22
PKI 6-22	91	53	0.9911	1.7	7.4
staurosporine	28	16	0.9708	5	15

^a Goodness of fit and reference values.

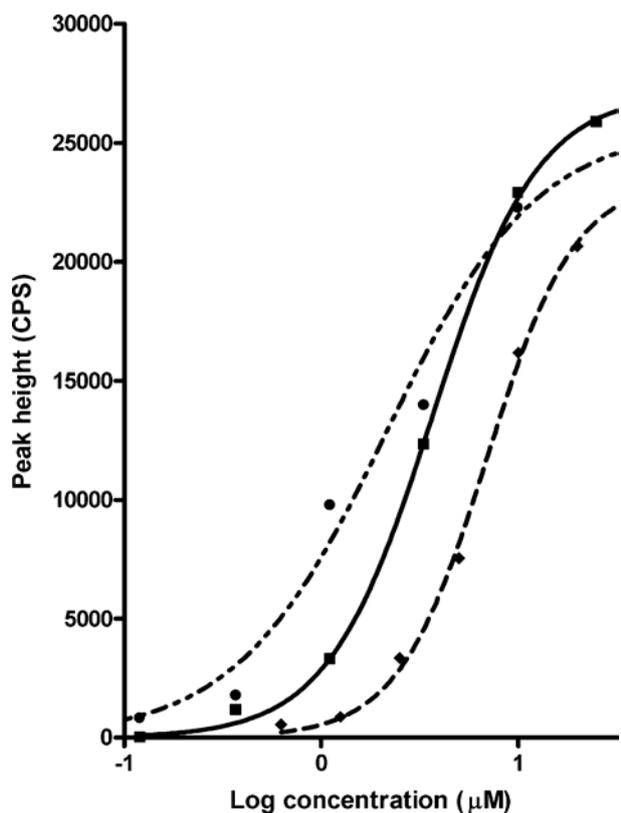


Figure 7. IC₅₀ curves. Solid line, squares PKI 5-24; dashed line, diamonds PKI 6-22; dashed line, circles staurosporine.

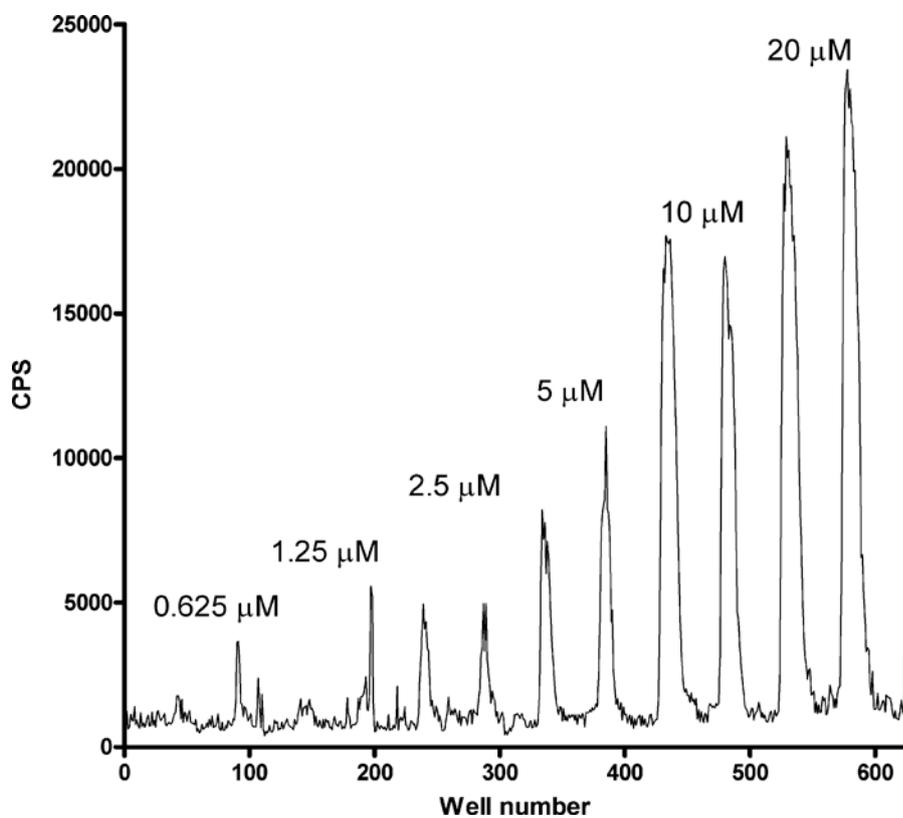


Figure 8. Biochemical trace - IC₅₀ determination for PKI 6-22. The shown concentrations refer to the injected solutions.

Conclusion

The presented technology provides two novel methodologies which were combined to give the final setup. First there is the online addition of all necessary biochemicals which is accomplished by two syringe pumps. This alone avoids any highly expensive liquid handling devices, such as pipetting robots or acoustic liquid handling procedures, making the described miniaturized 1536 well assay format accessible for equipment usually present in an analytical laboratory. Furthermore with the online addition step we also circumvent mixing and drying issues. This can be a problem particularly when working in a highly miniaturized 1536 well format. The second important development is the 1536 well fractionation technique itself with a fraction time of 2.5 s. As presented here it is possible to obtain an enzyme activity chromatogram when compiling the information of each micro well as a data point. This gives access to the full capabilities of today's plate reader technology, but furthermore the high resolution achieved by a HPLC separation is not compromised by the reaction time chosen. This should also allow the application of the procedure to a large number of enzymatic assays with the restriction that the employed assay has to be capable of tolerating some amount of organic modifier. The organic modifier content of the reaction buffer remains a problem for cell based assays, especially when human cell lines are employed. A reduction of the organic modifier content could possibly be accomplished by the application of higher separation temperatures or even High Temperature LC (HTLC). Finally the very fast fractionation times that can be achieved (down to 1.5 s) may allow the coupling of the microfractionation technology to ultrahigh pressure separations.

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Supplementary material

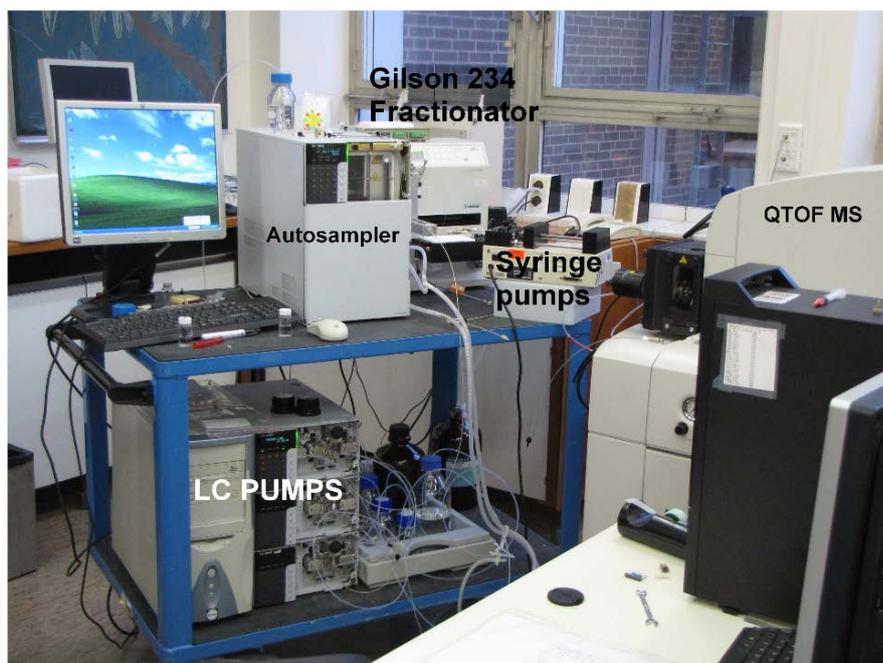


Figure S1. overview of the described system showing the main parts of the setup.

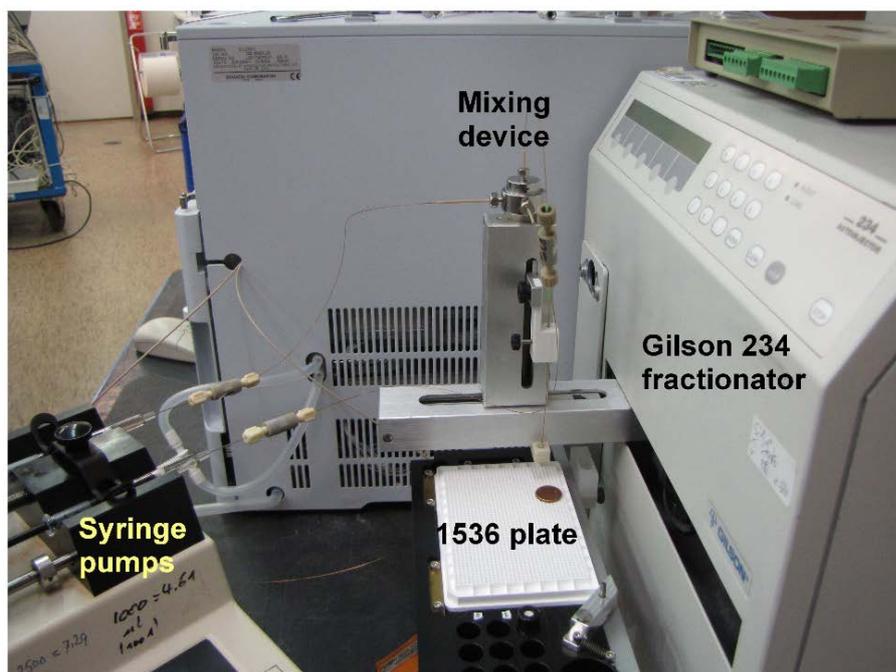


Figure S2. more detailed overview, showing a closer look of the developed system. The capillaries from the syringe pumps to the mixing device and the “fractionation” capillary from the mixing device to the plate were 150 μm ID fused silica capillaries, the capillary directing the flow towards the bioassay was a 50 μm ID fused silica capillary, all other used tubing was natural PEEK. The Gilson 234 autosampler served as sample processing robot, as can be seen in the video S6.

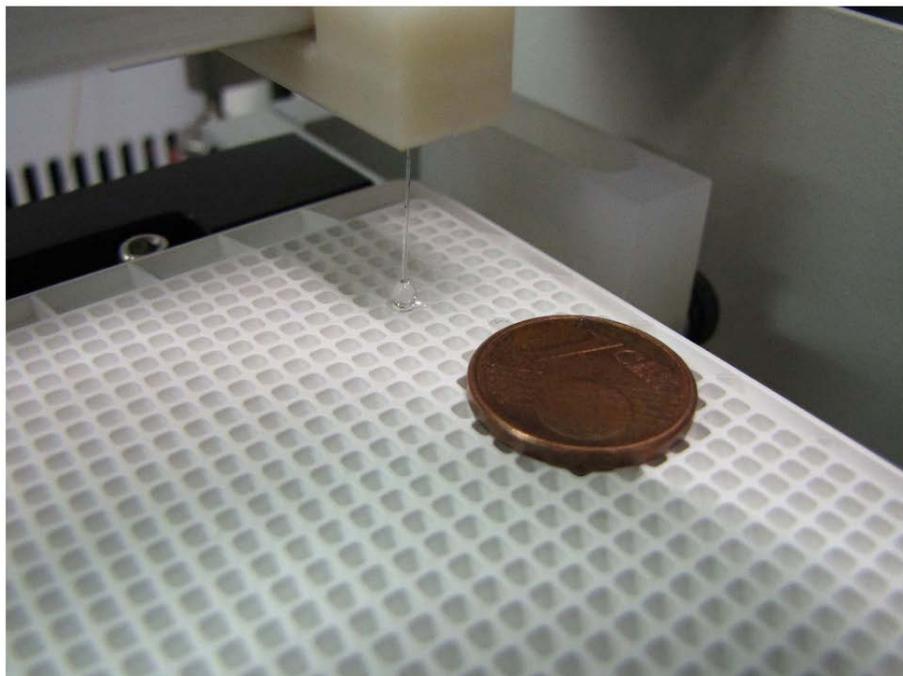


Figure S3. close look to the 150 µm ID fractionation capillary. The polyimide coating of the capillary was removed by “burning” it off, over an open flame. After the coating is removed the capillary is silylated as described and flushed with water before use.

S5 Calculation of the dilution factor

The effective concentration of the inhibitors in the at-line assay has to be corrected for a dilution factor, to make the results comparable with plate reader values. First the injected concentration of the inhibitors in the eluent flow (6 µL/min) is diluted with 44 µL/min caused by the reagent addition, this is resulting in a dilution factor of: 8.33. Second the peaks undergo longitudinal diffusion in the flow what also results in a dilution of the inhibitors. This factor (D) can be calculated by the following equation:

$$D = \frac{FWHM}{2} \cdot \sqrt{\frac{\pi}{\ln 2}} \cdot \frac{u}{V_i}$$

With an average full width at half maximum (FWHM) of 0.72 min, a flow rate (u) of 60 µL/min and an injection volume (Vi) of 5 µL this results in an dilution factor D of: 9.2. Taken together the overall dilution factor is 76.6.

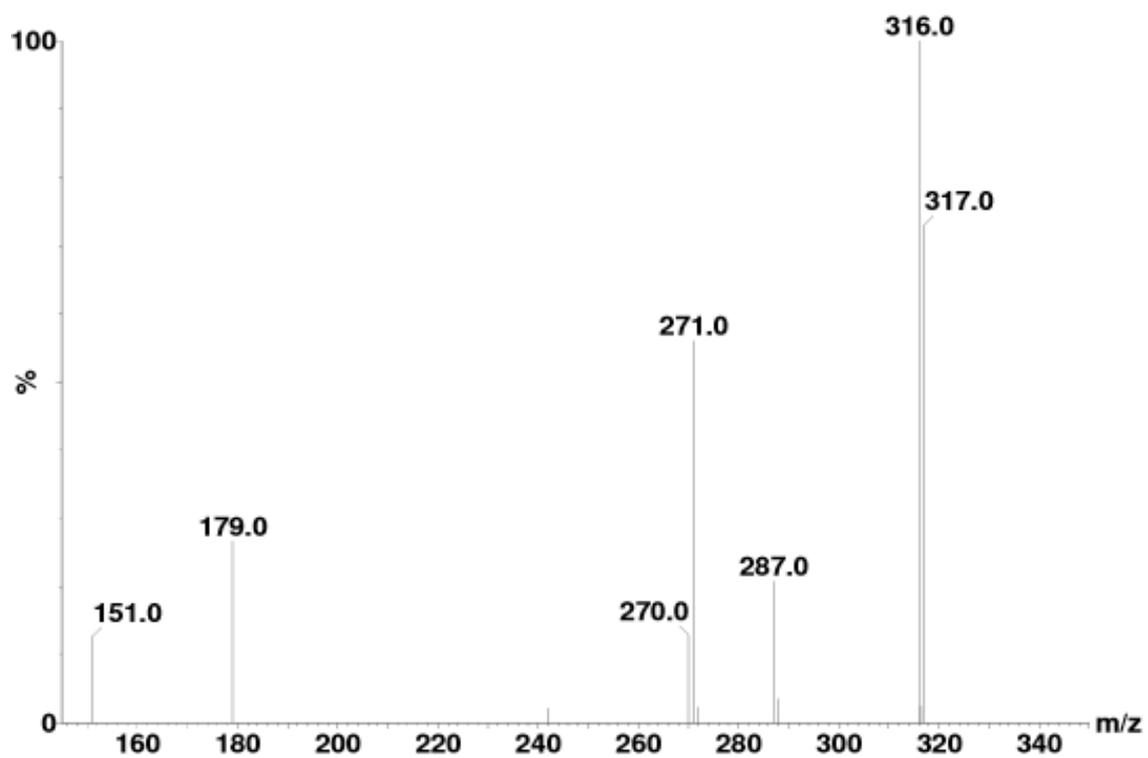
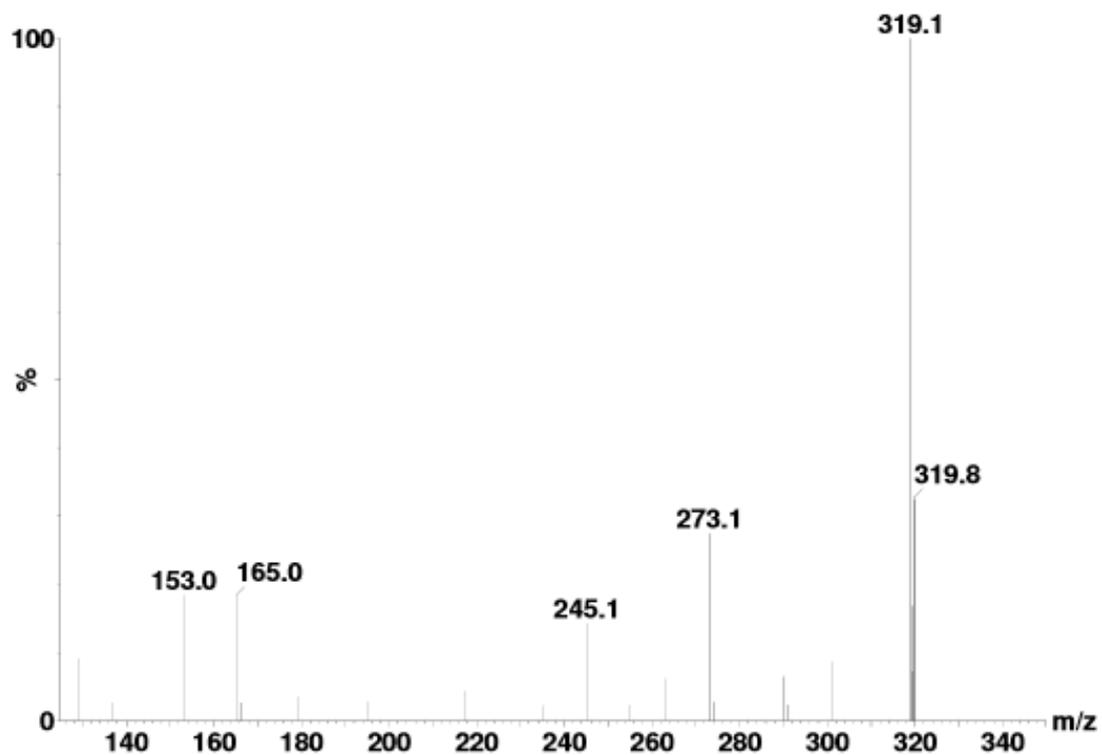


Figure S4. MS/MS spectra of Myricetin-3-O-rhamnoside above ESI+ spectra, isolated ion 465 m/z , collision energy 30 V below ESI- spectra, isolated ion 463 m/z , collision energy 30 V