Screening for bioactive co

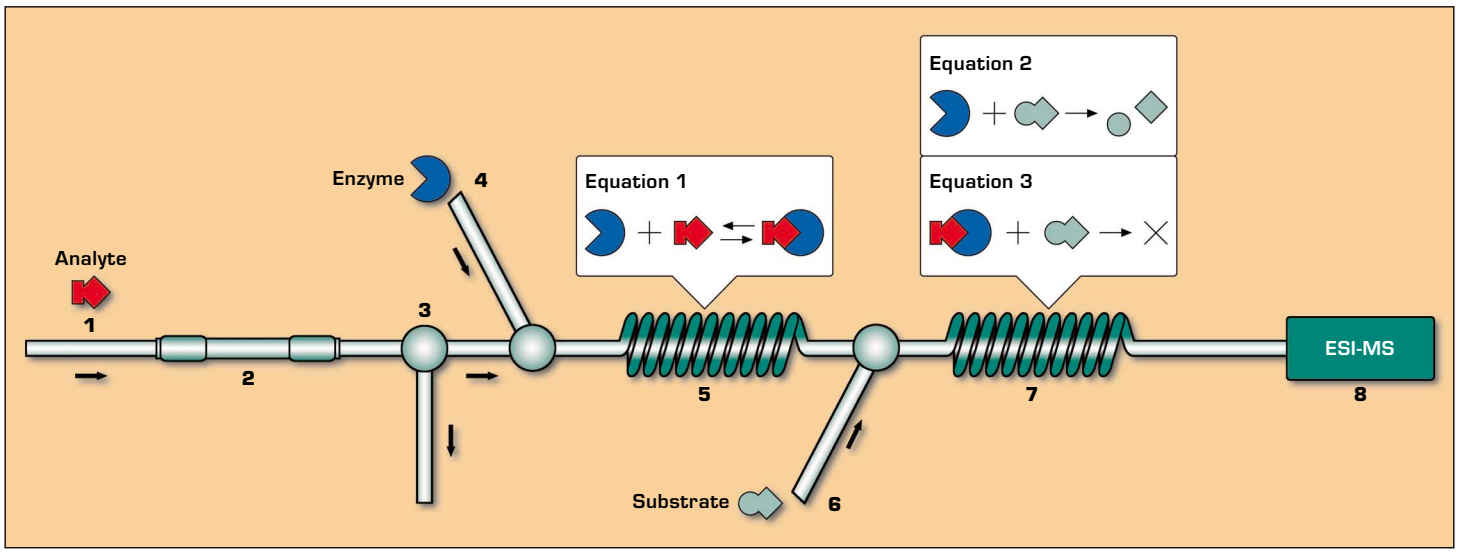

Figure 1: Schematic of the online continuous-flow system: **(1)** sample introduction via the carrier solution; **(2)** HPLC column; **(3)** 1:3 flow splitter, 50 µL/min bioassay:150 µL/min waste; **(4)** enzyme solution; **(5)** reaction coil A; **(6)** substrate solution; **(7)** reaction coil B; **(8)** LCMS-2010 mass spectrometer.

Enzyme converts the substrate continuously into products **(equation 2)**, if no bioactive compounds are eluting from the column. Bioactive compounds present in the eluate bind to the enzyme **(equation 1)**, resulting in a decrease of product turnover **(equation 3)**.

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Drug discovery often begins with the screening of synthetic and natural sources. High-throughput screening (HTS) technologies have been developed to test thousands of compounds per day from these sources for their activity in receptors or enzymes.

Although HTS techniques are highly efficient in the screening of pure compound samples, screening of complex mixtures is more demanding, involving a close coordination between chemical analysis, sample fractionation and biological screening. The simultaneous generation of both biological and chemical characteristics such as bioactivity, selectivity, concentration and molecular mass would be an advantage. This is demonstrated in the approach described, searching for enzyme inhibitors in complex mixtures such as natural product extracts. The system used consists of HPLC coupled to a continuous-flow enzymatic assay and the Shimadzu LCMS-2010 mass spectrometer, as depicted in Figure 1.

The continuous mixing of enzyme and substrate in reaction coil B results in a continuous flow of reaction products, directed to the mass spectrometer. The reaction products formed are used as reporter molecules for bioactivity determination. The enzymatic activity decreases if bioactive compounds bind to the enzymes in reaction coil A, thereby inhibiting the enzyme activity. As the enzymatic activity is proportional to the concentration of product formed, negative peaks in the product mass chromatograms expose bioactivity. Figure 2 illustrates how bioactive compounds are revealed in a flavonoids mixture.

Peaks reveal bioactive compounds

In general, the total ion current (TIC) does not provide any information regarding bioactivity. The mass chromatograms of the two products formed, Figure 2A and B, both reflect the biological activity of the system. In this example, two negative peaks showed up in these traces, indicating the presence of two bioac-

mpounds by means of ESI-MS

tive compounds. Mass spectra of the negative peaks are obtained (Figure 2C and D), showing the compounds eluting at this time. For molecular mass identification these peaks are plotted as extracted ion current chromatograms (Figure 2E and F). Bioactive peaks need to have the same retention time and peak shape as the negative peaks. In this example, the bioactive compounds identified in the mixture have the m/z of 358 and 427, respectively. To verify that the negative peaks were caused by bioactivity and not the result of system instabilities and/or ion suppression, two system monitoring compounds are added to the bioassay solutions.

The efficiency of the screening process was determined by the acquirement of IC_{50} values. The IC_{50} values obtained correspond closely with data reported by on fluorescence detection based assays. Detection limits measured for four bioactive compounds were well below the typical concentrations (1 - 10 µmol/L) used in HTS. Interday precision of the continuous-flow enzyme assay was 12.6 %, indicating that the methodology can be operated in a reliable way.

Biological and chemical characteristics and structural information

The main advantage is the generation of both biological and chemical characteristics such as bioactivity, selectivity, concentration, molecular mass and the possibility to obtain some structural information. The strong time correlation between the peaks of the reaction products and inhibitors allows the efficient assignment of molecular masses to the active compounds. Using retention time matching and peak shape comparison, even closely eluting compounds can be properly deconvoluted.

Conclusion

In comparison with enzyme assays based on fluorescence detection or colorimetric detection, the present approach has the distinct advantage that no synthesis efforts are required to prepare a fluorimetric substrate with the desired detection properties while retaining sufficient affinity for the enzyme.

References

A.R. de Boer, T. Letzel, D. A. van Elswijk, H. Lingeman, W.M.A. Niessen und H. Irth. Anal. Chem. 76, 3155-3161, 2004

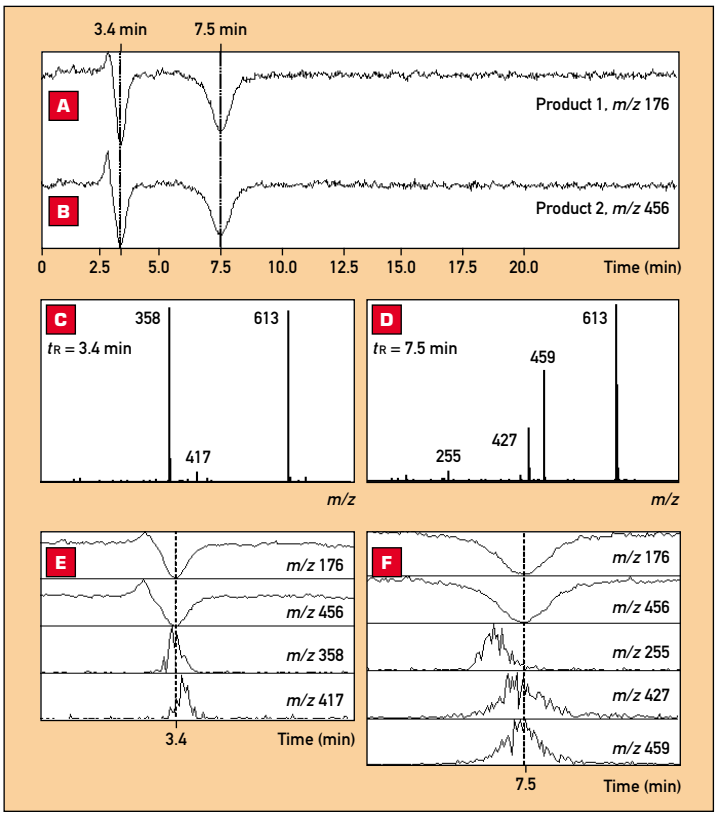

Figure 2: Online HPLC continuous-flow experiment of a mixture of flavonoids containing two bioactive compounds. **(A)** mass chromatogram of product 1; **(B)** mass chromatogram of product 2; **(C)** mass spectrum recorded at t_R = 3.4 min; **(D)** mass spectrum recorded at t_R = 7.5 min; **(E)** RICs of the most abundant peaks shown in the mass spectrum recorded at t_R = 3.4 min; **(F)** RICs of the most abundant peaks shown in the mass spectrum recorded at t_R = 7.5 min.