

Automatic SPE Extraction of Red Wine for NMR: A New Tool for Chemometrics and Hyphenation

Solid phase extraction (SPE) is a well-known sample preparation method for many analytical techniques. It is used in environmental, pharmaceutical, food and natural products applications for sample clean-up and pre-concentration of less polar analytes. Typically, it is used in combination with separation techniques and mass spectrometry (LC-MS, GC-MS) for quantitative analysis of trace components present in aqueous samples.

Due to the separation of highly polar matrix constituents from less polar trace analytes and the increase in concentration, SPE is said to be of great benefit in the NMR analysis of water-based food samples such as juice and wine ^[1,2].

Secondary plant metabolites enriched by this technique and detected by NMR play a key role in the differentiation of grape varieties. The submission of the extract to hyphenated techniques such as LC-MS or LC-NMR enables the identification and structure elucidation of differentiating markers.

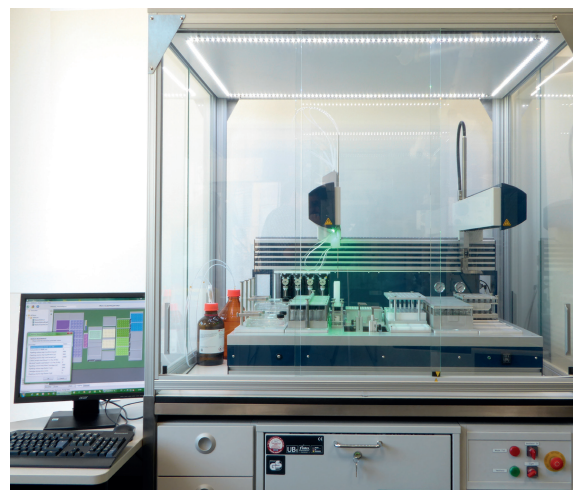
Materials and Methods

Automated Solid Phase Extraction

10 mL of authentic wine samples from primitivo and corvina grapes were directly subjected to an automated SPE device (Bruker BioSpin, Rheinstetten, Germany) shown in Figure 1. After condition with 5 mL of methanol and equilibration with 5 mL of deionized water, the samples were transferred onto 3 mL cartridges filled with 200 mg of polymer based LiChrolut EN SPE material (Merck Millipore, Darmstadt, Germany).

During this loading step, fractions of matrix components not retained on the SPE material were collected in 12 mL vials for NMR analysis of highly polar plant metabolites. After washing with 5 mL of deionized water and 20 min drying with nitrogen gas, the less polar constituents are eluted with 3 mL of methanol, prepared with 5% CD₃OD for the NMR lock and 0.02% TSP as chemical shift reference. After mixing, 1000 µL of each extract is transferred into a 5 mm NMR tube sealed with a septum cap.

Figure 1



Automatic SPE device SamplePro SPE from Bruker BioSpin.

The original sample and the load fraction were prepared by mixing with NMR buffer and adjusting the pH before transfer in a 5 mm NMR tube.

NMR

NMR spectra are recorded on a FoodScreener™ (Bruker BioSpin, Rheinstetten, Germany) acquiring NMR spectra with 8-fold (original sample, load fraction) and 2-fold (methanol extracts) solvent suppression using a modified 1D-NOESY sequence in full automation.

Statistical Analysis

NMR raw data are subjected to non-supervised statistical principle component analysis (PCA) using AMIX software (Bruker, Rheinstetten, Germany).

LC-SPE-NMR/MS Analysis

After identification of differentiating resonances for primitivo wines, 3 pooled extracts showing the highest concentrations of the marker were evaporated to dryness and re-dissolved in 100 µL methanol.

5 x 10 µL of this extract was injected on a 250 x 4.6 mm 5 µm Kinetex C18 column (Phenomenex, Aschaffenburg, Germany) using an injector program.

Chromatography was performed using an Agilent 1200 system consisting of a quaternary pump, autosampler, column oven and diode array detector. Mass spectrometric detection was conducted using a MicroTOF (Bruker Daltonics, Bremen, Germany) operated in negative electrospray ionization mode with a scan range from m/z 50-1000.

Separation was achieved with methanol-water (each 0.1% CDOOD) as mobile phase with a flow rate of 0.5 mL/min. The elution gradient was: 0 min, 2% methanol; 5 min, 2% methanol; 50 min, 60% methanol; 55 min, 100% methanol.

Post column solid phase extraction was performed in time slice mode on a Spark Prosekt2 (Emmen, The Netherlands) with a water make-up flow of 1.5 mL/min on 10 x 2 mm Hysphere GP resin cartridges.

The cartridges were transferred with CD₃OD to an AVANCE III 500 MHz NMR spectrometer equipped with a 5 mm TCI Prodigy Cryo Probe. A 60 µL cryofit converted the Cryo Probe into a flow probe connected to the Prosekt2 SPE device. After NMR measurement, the extracts were recovered in cryo vials for further clean-up of individual compounds and structure elucidation.

For clean-up, the recovered fractions were evaporated to dryness and reconstituted in 50 µL methanol. The fraction was then injected on the column described above. Instead of methanol, the chromatography was performed with acetonitrile, in order to change the selectivity of the separation and hence isolate the pure compound for further structure elucidation.

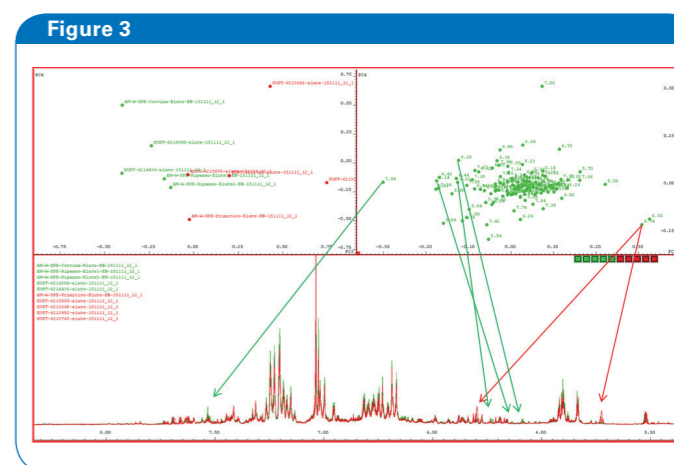
Clean 1D and 2D NMR spectra together with the molecular formula were analysed with CMC-se software (Bruker Biospin, Rheinstetten, Germany) for structure generation.

Results and Discussion

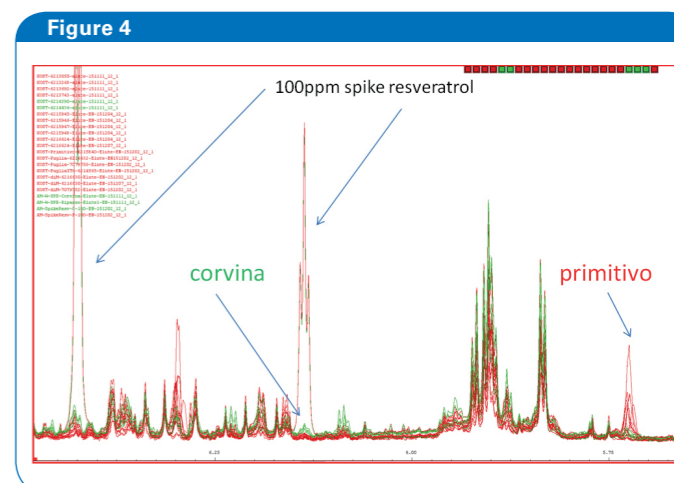
Figure 2 shows the separation of the matrix components from the secondary plant metabolites. This is shown by comparing the NMR profiles of the standard preparation, preparation of the load fraction and the NMR spectrum of the SPE extract for a corvina and a primitivo wine.

Beside the increase of the signal-to-noise ratio in the aromatic and olefinic region of the NMR spectrum due to the enrichment step, the depletion of polar matrix constituents (e.g. anomeric protons of glucose) can be observed clearly. The NMR spectra of the SPE extracts of both wines reveal slight differences in the aromatic and olefinic pattern.

Statistical analysis of a larger set of samples identifies chemical shifts discriminating between both wine types, depicted in Figure 3. Standard addition of resveratrol, a compound known to be present in wine, identifies one possible marker in corvina due to elevated concentrations (Figure 4), while other resonances remain unknown.

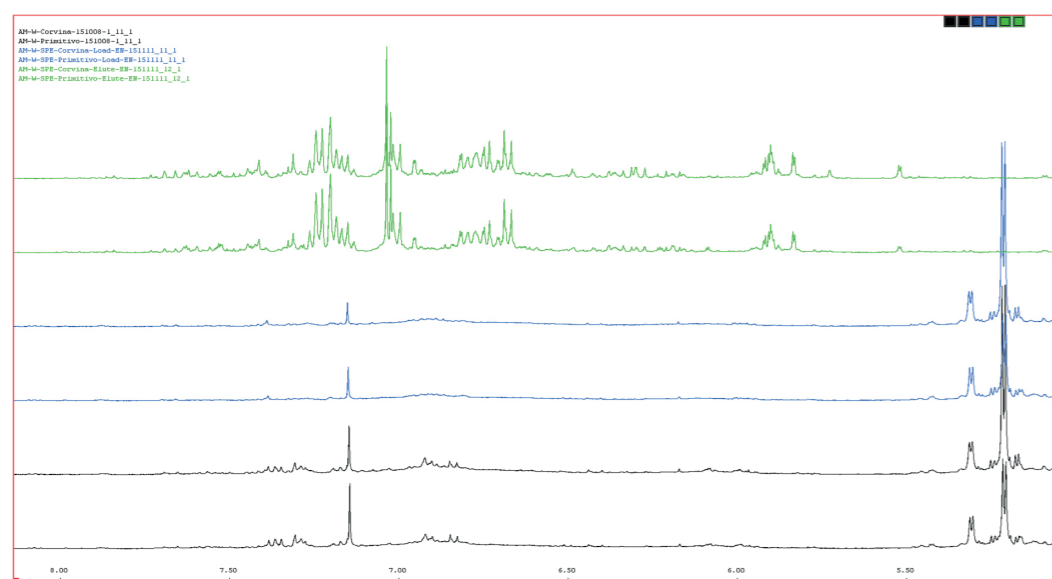


PCA plot of 5 corvina (green) and 5 primitivo (red) wine SPE extracts.



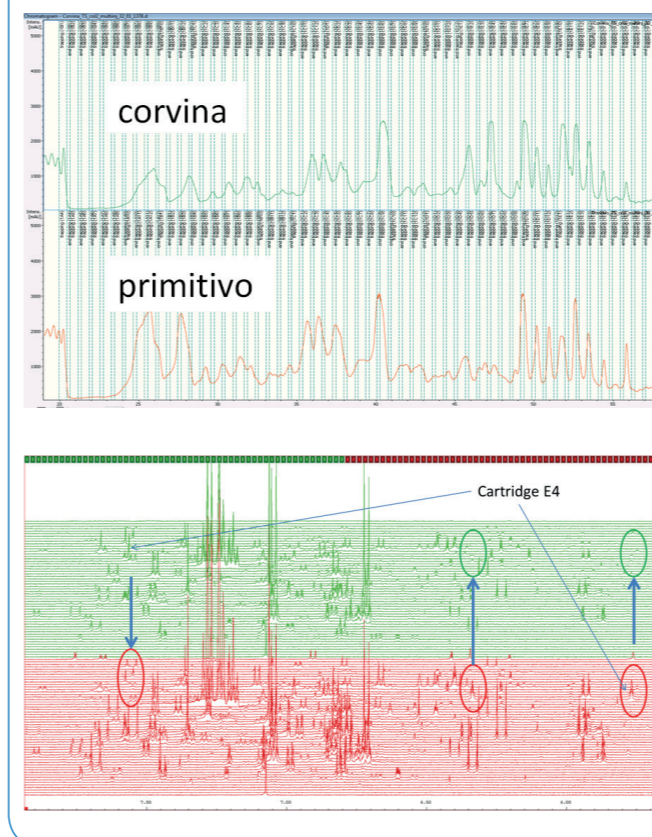
Overlay of NMR spectra of corvina (green) and primitivo (red) wine SPE extracts. Two samples were extracted after standard addition of 100ppm resveratrol.

Figure 2



NMR spectra (aromatic region) after standard preparation of corvina and primitivo wine (black), the load fraction (blue) and the SPE extract (green).

Figure 5



Time-slice NMR chromatogram after collecting 43 sec intervals (55 fractions) of the LC-UV chromatogram shown above. Resonances of discriminating markers are labeled in the NMR chromatogram. Green: corvina, red: primitivo.

Non-Targeted LC-NMR Hyphenation

The identity of the unknown marker cannot be established by library search if the compounds are not commercially available. The only way to identify the compounds is by isolation and structure elucidation after extracting information based on MS and NMR spectroscopic data evaluation. As only information about chemical shifts is known, classical UV or MS based peak collection schemes cannot be used. Instead, the so-called time slice technique can be employed, where pre-fixed time fractions of the chromatogram are trapped on post column SPE cartridges. These cartridges are subsequently transferred to the NMR and recovered after acquisition. If the compound of interest is present in the extract, the time fraction can be re-injected to the HPLC column for a clean-up as described above. During this step, parallel MS detection delivers the molecular formula of the unknown, while NMR provides all necessary data for structure elucidation, as shown in Figure 5.

Using this technique, the pre-isolation of unknowns can readily be achieved. Further cleaning and structure elucidation using CMC-se leads to the structures shown in Figure 6.

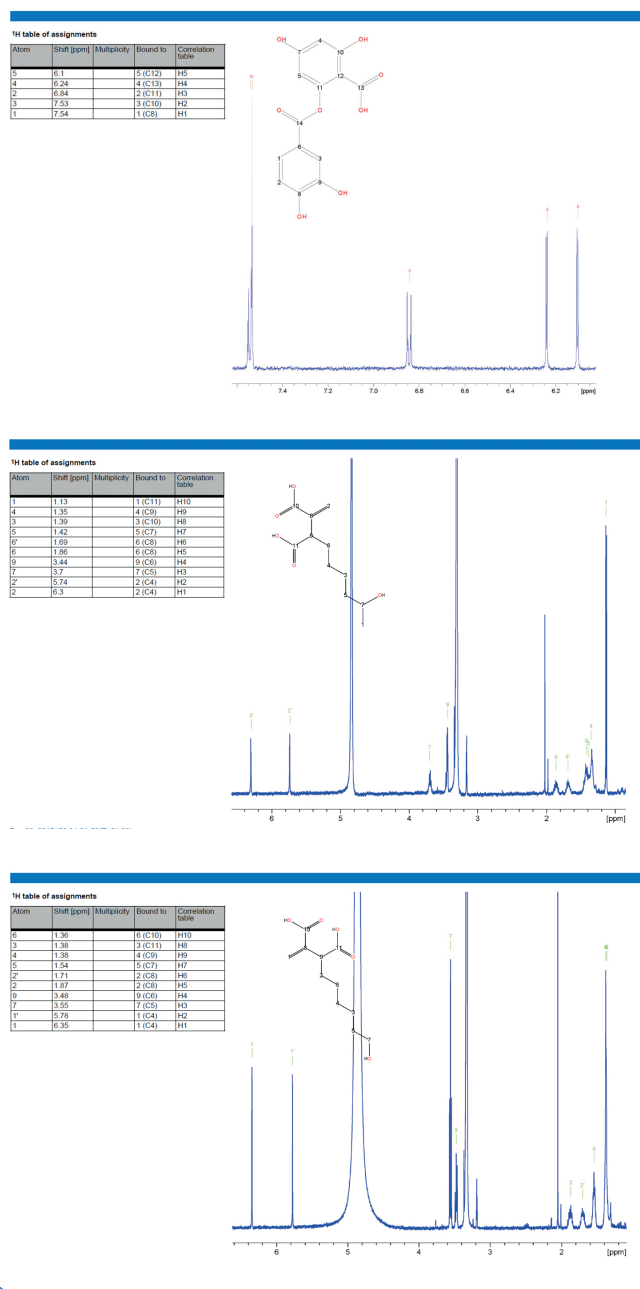
Conclusion

SPE-NMR provides new insights into the metabolome of plants, as it increases the concentration of secondary plant metabolites while depleting unwanted small molecules, e.g. sugars, amino acids and small organic acids. Here, it is used for the discrimination of wines produced from different grapes. The same method can also be used for juices and other aqueous samples. Once markers are identified by statistical analysis, the LC-NMR technique can help to isolate and elucidate the structure of unknown markers.

References

- [1] Anastasiadi M, Zira A, Magiatis P, Haroutounian SA, Skaltsounis AL, Mikros E. ¹H NMR-based metabonomics for the classification of Greek wines according to variety, region, and vintage. Comparison with HPLC data. *J Agric Food Chem.* 2009 Dec 9;57(23):11067-74
- [2] Ali K, Maltese F, Fortes AM, Pais MS, Verpoorte R, Choi YH. Pre-analytical method for NMR-based grape metabolic fingerprinting and chemometrics. *Anal ChimActa.* 2011 Oct 10;703(2):179-86

Figure 6



Structures of preliminary marker for corvina (top) and primitivo (bottom) wines.