Effective On-Line Sample Clean-Up and Analyte Enrichment for UHPLC Analyses

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Overview

Purpose: Development of a robust, user-friendly, and fast on-line SPE-UHPLC method to improve the traditional off-line SPE-HPLC analysis of isohumulones in beer.

Methods: Several mobile phases, column chemistries, temperatures, and on-line SPE setups were used to investigate the best synergy of simplicity, robustness and chromatographic result.

Results: An on-line SPE method has been created which meets all requirements mentioned above. Compared to traditional off-line SPE-HPLC workflows which last an hour, this on-line SPE-UHPLC workflow is completed in less than ten minutes.

Introduction

Sample preparation is a crucial aspect in analytical chemistry, especially in the food and beverage applications with highly complex matrices. Different sample preparation techniques can be used to remove matrix or enrich analytes of interest. Solid Phase Extraction (SPE) is most often used as sample preparation prior to HPLC analyses. In general, SPE is a manual off-line procedure, hence a significantly time-consuming bottleneck, and also a source of error in the laboratory workflow. On-line SPE automates the sample cleanup and analyte enrichment process and therefore overcomes all issues mentioned above. Moreover, direct injection of untreated samples becomes possible and unattended operation a reality.

In this presentation, an automated on-line SPE-UHPLC solution is demonstrated by the separation of isohumulones in beer. Isohumulones are developed from the resins of hop (*Humulus lupulus* L.*),* and are generated during the boiling of wort, forming the typical bitterness of beer. Moreover, their antimicrobial effect leads to a sterile beverage, their tensioactive character stabilizes the foam, and they have a huge influence on the general flavor and smoothness of beer.[3,4] Hence, monitoring their content during and after the brewing process is mandatory to control beer quality. Traditional analyses last about an hour or longer due to manual sample pretreatment steps, off-line SPE, and a conventional HPLC separation.[2,3] With automated on-line SPE-UHPLC, an untreated beer sample is injected directly, all SPE-steps are performed automatically, and the entire analysis lasts only nine minutes.

Method

Sample Preparation

Frisian pilsener beer was injected without any sample preparation.

Mobile Phases and Chemicals

A: Water (Fisher Scientific Optima LC/MS) with 1.0% formic acid and 0.2 mM EDTA
B: acetonitrile (Optima™ LC/MS)

Thermo Scientific Dionex UltiMate 3000 x2 Dual RSLC with Automated On-Line SPE Setup consisting of:

Dual Gradient Pump (DGP-3600RS)

Left pump (loading pump) : 2.0 mL/min	Right pump (analytical pump): 0.65 mL/min
0 min: 90% A – 10% B	0 min: 50% A – 50% B
2 min: 90% A – 10% B	9 min: 50% A – 50% B
2 min: 0% A – 100% B	
4 min: 0% A – 100% B	
4 min: 90% A – 10% B	
9 min: 90% A – 10% B	

Autosampler (WPS-3000TRS)	Column compartment (TCC-3100RS)
Injection volume: 1–80 µL	Oven temperature: 30 °C
Tray temperature: 10 °C	

UV-Detector (VWD-3400RS)	2pos-6port HT valve (TCC-3100RS)
Flow cell volume: 2.5 µL	0.0 min: Position 1_2
Wavelength: 270 nm	1.5 min: Position 6_1
Detection collection rate: 25 Hz	2.0 min: Position 1_2

Stationary Phases

Analytical column: Thermo Scientific Hypersil GOLD, 1.9 µm, 2.1 × 100 mm
SPE column 1: Thermo Scientific SolEx HRP RSLC, 12–14 µm, 2.1 × 20 mm
SPE column 2: Thermo Scientific SolEx Acclaim C18 prototype, 5 µm, 2.1 × 20 mm

Data Analysis

Thermo Scientific Dionex Chromeleon 7.1 Chromatography Data System (CDS) with service release SR1 was used for data processing.

On-Line SPE-UHPLC Principle

FIGURE 1. Analyte extraction, matrix depletion, and equilibration of the analytical column.

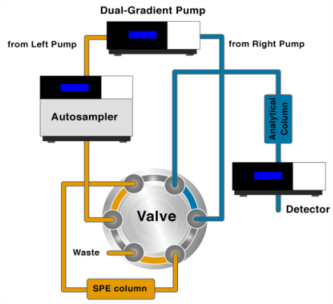
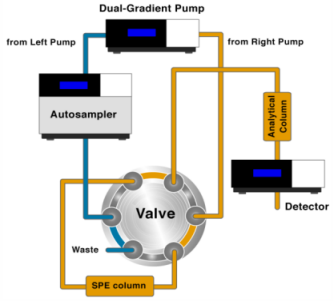


FIGURE 2. Analyte transfer from the SPE to the analytical column.



The figures above show the typical setup for on-line SPE-UHPLC. In the first step, the sample is injected to the SPE column with the use of the left pump (Figure 1). The sample matrix is flushed to waste while the analytes of interest are trapped on the SPE column. At the same time, the analytical column is equlibrated by the right pump.

After the matrix is flushed off the SPE column, the six-port valve is switched (Figure 2). This way, the SPE coulmn is coupled in series with the analytical fluidic path and the analytes are transferred to the analytical column in back-flush mode. After this transfer, the six-port valve is turned to the initial position of Figure 1. The analytes of interest are separated on the analytical column (right pump) while the SPE column is washed and equilbrated (left pump).

The time to elute the matrix and the time when the analytes of interest begin to elute from the SPE column must be determined to define the first valve switching time t_1. To acount for the best possible matrix depletion without loss of analytes, t_1 must be in between both times described above .

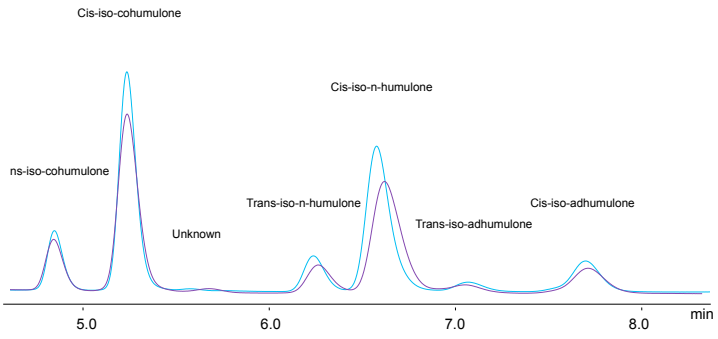
The second valve switch (t_2) has to occur when all analytes are completely transferred to the analytical column. It strongly depends on the analytical method whether t_2 needs to be before, or after, the analytical separation, especially in gradient elution.

Results and Discussion

Effect of Ethylenediaminetetraacetic acid (EDTA) as eluent component

Fully de-mineralized mobile and stationary phases proved to be mandatory, as even traces of metal ions reduce recoveries of isohumulones due to their extreme binding affinity towards metals.[3] Ethylenediaminetetraacetic acid (EDTA) masks potential metal ions and suppresses their interaction with isohumulones. The resulting decrease of peak area and therewith recovery rate for all isohumulones hampers LC analysis (Figure 3).

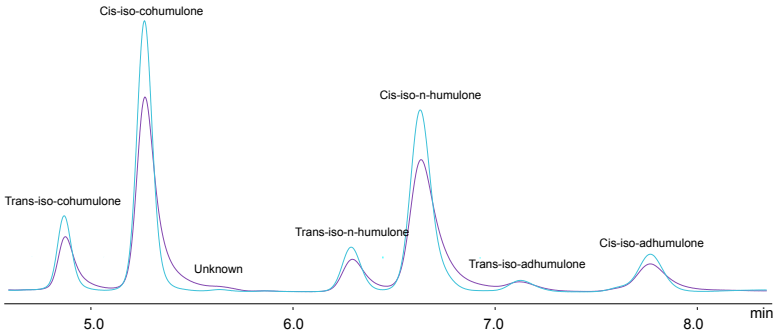
FIGURE 3. Mobile phase A without EDTA (violet) or with 0.2 mM/L EDTA (blue). Both chromatograms were derived by using the back-flush on-line SPE setup with the SolEx™ Acclaim™ C18 RSLC prototype SPE column and an injection volume of 40 µL beer sample each.



Influence of the stationary phase material of the SPE column

it was observed that the SolEx HRP material affected isohumulone peak shapes when compared to direct injections without SPE. This may be attributed to strong hydrophilic interactions of this mixed-mode polymer. This adverse effect was not encountered with a silica based C18 stationary phase packed into the SolEx RSLC SPE column hardware.

FIGURE 4. Comparison of SolEx HRP RSLC (violet) and SolEx Acclaim C18 RSLC prototype (blue) SPE-columns. Both chromatograms obtained from on-line SPE back-flush after injection of 40 µL beer sample.



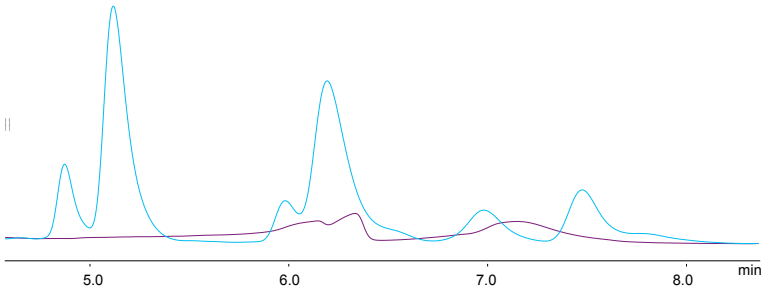
Using Acclaim C18 silica instead of the HRP polymer as stationary phase of the SPE column, all peaks became baseline-separated and an unknown could be seperated from the cis-iso-cohumulone (Figure 4).

It is vey important for a brewer to know about the actual composition of isohumulones in the beer to guarantee a balanced bitterness, smoothness, taste, and stability. For example, iso-cohumulone is associated with hop quality but also shows the harshest bitterness of all isohumulones. Iso-adhumulones are the most stable isohumulones and cis-isomers are generally more stable than the trans-isomers[3]. The efficient baseline separation completely fulfills the requirements for accurate quantitation of each iso-humulone.

Back-flush vs. fore-flush analyte transfer from the SPE column

Next to the commonly used back-flush mode, on-line SPE can also be done in a fore-flush mode. In fore-flush mode, both loading and analytical pump feed the SPE column in the same flow direction. This was accomplished by swapping the plumbing positions of valve to analytical column and valve to analytical pump (see Figure 1).

FIGURE 5. Comparison of SolEx HRP RSLC (violet) and SolEx Acclaim C18 RSLC prototype (blue) SPE-column. Both chromatograms obtained from on-line SPE fore-flush after injection of 40 µL beer sample.



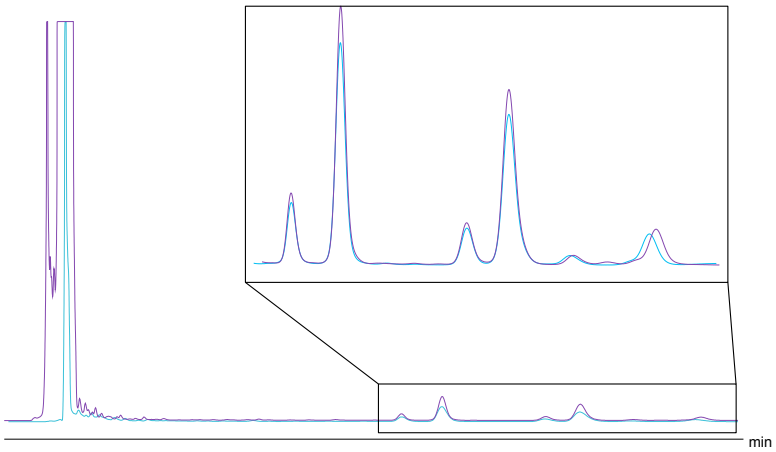
It is described in literature that different types of C18 columns have a significant influence on selectivity and resolution of isohumulones [3]. While fore-flush provides perfect filtering to protect the analytical column, its inherent sequential application of columns with different selectivities is prone to influence the resulting separation.

Figure 5 demonstrates the impact on the separation when Acclaim C18 silica was used as SPE column with fore-flush. With the HRP polymer stationary phase SPE column, the separation of isohumulones was totally disrupted in fore-flush mode. This clearly dictated back-flush as the operational mode for this application.

Recovery rate and general comparison of direct injection with on-line SPE

The removal of sample matrix is the most substantial argument for applying on-line SPE. While removing the sample matrix, analytes of interest should be completely retained on the SPE column.

FIGURE 6. Comparison of a direct injection without SPE column (violet) and an on-line SPE using the SolEx Acclaim C18 RSLC prototype (blue) in back-flush mode (injection volume of 50 µL beer sample each, times normalized and not shown).

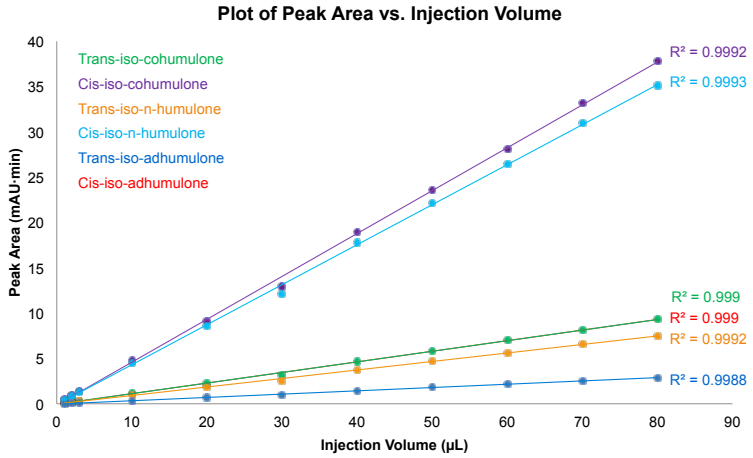


The main part of the beer matrix was excluded from the analytical column with on-line SPE (Figure 6). While the initial signal was out of range with a direct injection, the on-line SPE chromatogram showed only a small matrix peak. The recovery rate of each isohumulone was found to be ≥ 95% and the impact on the resulting separation was minor.

Linearity and detection range

Different volumes of beer (from 1 µL up to 80 µL) were injected on the on-line SPE setup by using the back-flush mode. The analysis of each injection volume was repeated three times. The average value of those three results was plotted (Figure 7).

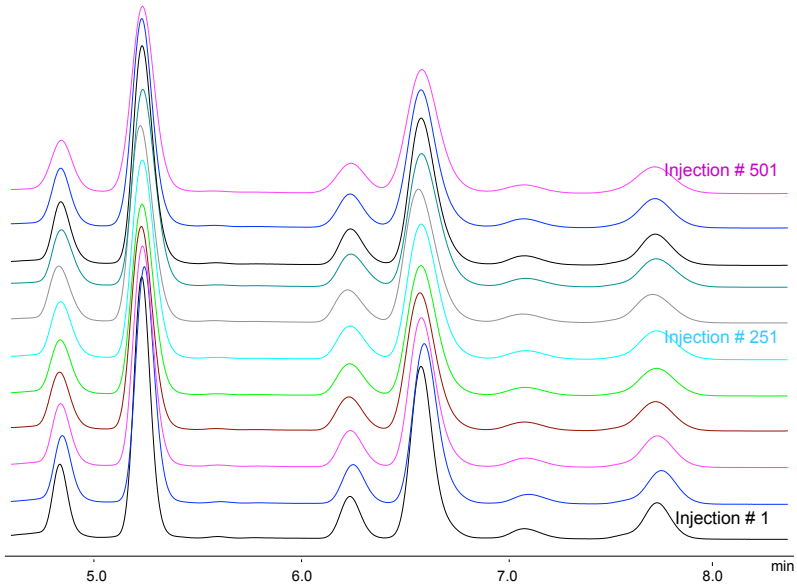
FIGURE 7. Plot of peak areas resulting from different injection volumes of the same beer sample with on-line SPE (back-flush mode) using the SolEx Acclaim C18 RSLC prototype.



Depending on beer type and brand, the total concentration of isohumulones is reported to commonly range from 10 mg/L up to 100 mg/L[1,3], while the Frisian Pilsener beer used for our experiments should contain about 40 mg/L of isohumulones in total[1]. Even the injection of 1 µL beer allowed to evaluate the peak area of the isohumulone with the lowest concentration (trans-iso-adhumulone). Relative peak areas of each isohumulone (trans-iso-cohumulone 9%, cis-iso-cohumulone 37%, trans-iso-n-humulone 7.5%, cis-iso-n-humulone 35%, trans-iso-adhumulone 2.5%, and cis-iso-adhumulone 9%) were found to be constant for all used injection volumes, as the linear regression proves (Figure 7).

Reproducibilty and long-term stability

FIGURE 8. Endurance test of on-line SPE in back-flush mode using the SolEx Acclaim C18 RSLC prototype (injection volume of 40 µL beer sample each).



The long term stability of the method and the SPE-coulmn is shown in Figure 8. With continuous operation, 510 injections were made before the SPE-column became blocked,. and therewith exceeded the upper pressure limit of the loading pump. The separation efficiency did not suffer significantly and the relative peak area of each isohumulone was found to be constant for all injections.

Conclusions

- EDTA is required in mobile phase A to mask residual metal ions.
- Excellent separation efficiency and resolution of isohumulones was achieved with the SolEx Acclaim C18 prototype rather than SolEx HRP.
- The back-flush mode lead to superior results compared to the fore-flush mode.
- The recovery rate of the back-flush on-line SPE setup was between 95% and 100% when compared to a direct injection.
- The linearity between injection volume and detector response was proven for all isohumulones.
- The analysis was very robust and the SPE-column withstood 500 injections of a pilsener beer sample.
- The entire on-line SPE workflow needed less than ten minutes whereas off-line SPE workflows required at least one hour.

References

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