Application Note

Preparative Isolation of Piperine from Black Pepper Extracts

Category	Food analysis
Matrix	Pepper Extract
Method	analytical HPLC, preparative HPLC
Keywords	Pepper, quality control, purity control, method development, Vertex Plus AX preparative column, AZURA Compact, AZURA Prep
Analytes	Piperine
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Summary

This application note presents how AZURA compact and AZURA preparative HPLC systems work together hand in hand in one whole process of the method development, purification and purity control of Piperine from black pepper extracts. Applying a Eurospher II C18 preparative column with axial compression technology leads to a robust and reliable preparative HPLC method for the fractionation of Piperine. The BlueShell classic C8 core shell column shows at the same time highest resolution for the subsequent purity control on a classic and cheap HPLC system with moderate backpressure.

Introduction

Black pepper (*piper nigrum*) is a widely used hot spice. Furthermore it is used in the traditional medicine[1]. Piperine (Fig. 1) is the main compound leading to bioactivity of black and white pepper. His pungency has been estimated as 100000-200000 Scoville Unit [2, 3].

Piperine is an alkaloid and it is the carboxamide of Piperic acid and Piperidine. It shows low solubility in water, but ethanol and other organic solvent are suitable for solving this substance. In recent decades, Piperine came into the focus of pharmaceutical research. It has antibacterial[4], antioxidant[5], anti-inflammatory[6], antiarthritic[6] and other effects. The most interesting point is that Piperine increases the bioavailability of a number of therapeutic drugs as well as phytochemicals[7].

The concentration of Piperine in black and white pepper ranges only from 3 to 8 %[8]. Therefore a purification step is required for the recovery of Piperine from natural pepper. In this application note the acetone extraction of Piperine from black pepper, the isolation of this compound by preparative chromatography and furthermore the corresponding quality analysis will be shown.

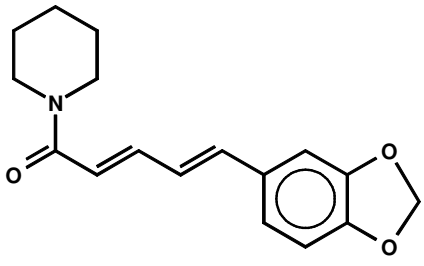


Fig. 1

Chemical structure of Piperine (trans, trans form)

Experimental sample preparation

Piperine was extracted from Pepper using acetone. Therefore, approx. 6g grounded black pepper was exactly weighed in a suitable flask and 50 ml Acetone (p.A.) were added followed by 30 min ultrasonic bath at 60°C. After a total extraction time at 60°C of about 120 minutes, the powder was sedimented and the supernatant was collected. While caring that no precipitation occurs in the sample 600 µl water were added per 1 ml extract. After filtration through a 0.45 µm filter, the solution was ready for injection to the HPLC system respectively the purification via preparative HPLC.

The screening method was developed with the aim to separate the target peak Piperine from the matrix. At the same time the method has to be robust and cheap, because it will run in the preparative mode and consume a lot of eluent after the scale up step. The column also has to be up-scalable to the prep range. Under these precautions a Eurospher II C18 column with 5 µm particle size was chosen and a water acetone mixture was used as the mobile phase during analytical method development. Acetone is not commonly used as an eluent in HPLC, but caused by the much cheaper price compared to acetonitrile or methanol, it was in this case chosen as the mobile phase. Furthermore it is a very volatile liquid what will simplify the recovery of the purified Piperine out of the preparative mobile phase.
The resulting method was transferred to the preparative range keeping the stationary and mobile phase identic and scaling up the method parameters. The scale up factor is 25 for scaling up from a 4 mm ID column up to a 20 mm ID column filled with the same column material.

After purification of Piperine via preparative chromatography, the pureness of the fraction was investigated with a different HPLC method. In this case, the BlueShell classic C8 column was chosen, because of its high resolution. The core shell technology used for these particles leads to really sharp peaks and at the same time tolerable backpressure. For the purity control, acetonitrile was used as the mobile phase because here the aim was to get the highest resolution and not the cheapest analysis.

Method parameters

Analytical method for scale up to preparative HPLC

Column	Eurospher II 100-5 C18, 250 x 4 mm ID with integrated precolumn
Eluent A	Water
Eluent B	Acetone
Gradient	Isocratic 60 % B
Flow rate	1.0 ml/min
Injection volume	2 µl (resp. 40 µl for overloading)
Column temperature	25 °C
Detection	UV at 210 nm and 340 nm, 10 mm flow cell, 20 Hz, 0,05 s
Run time	14 min

Preparative method for purification of Piperine

Column	Eurospher II 100-5 C18, 250 x 20 mm ID axial compression with precolumn 30 x 20 mm
Eluent A	Water
Eluent B	Acetone
Gradient	Isocratic 60 % B
Flow rate	25.0 ml/min
Injection volume	appr.. 1000 µl (via pump)
Column temperature	25 °C
Detection	UV at 340 nm, 3 mm flow cell, 5 Hz, 0,02 s
Run time	14 min

Analytical method for purity control

Column	BlueShell classic 80-4.5 C8 core shell, 150 x 3 mm ID
Eluent A	Water
Eluent B	Acetonitrile
Gradient	Isocratic 30 % B
Flow rate	1.0 ml/min
Injection volume	5 µl
Column temperature	25 °C
Detection	UV at 201 nm, 10 mm flow cell, 20 Hz, 0,05 s
Run time	20 min

Results

The first analysis was run to screen for the right column and the best fitting method parameters for preparative HPLC. Fig. 2 shows the corresponding chromatogram recorded with the optimized analytical method. The blue trace shows the chromatogram at 340 nm and the green trace at 210 nm. The target peak shows highest abundance at 340 nm and therefore this wavelength was also chosen in the preparative scale. On the other hand, most impurities are best detectable at 210 nm. This becomes obvious from the baseline zoom-in. in fig. 2. This is also the reason why purity control is recommended at 210 nm.

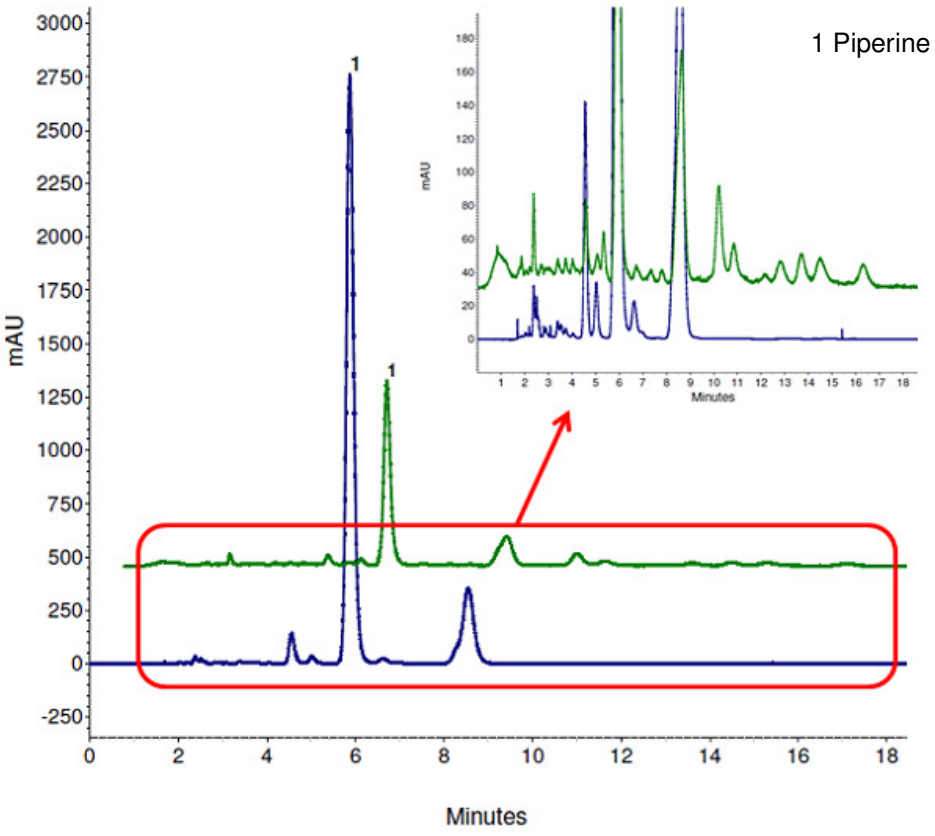
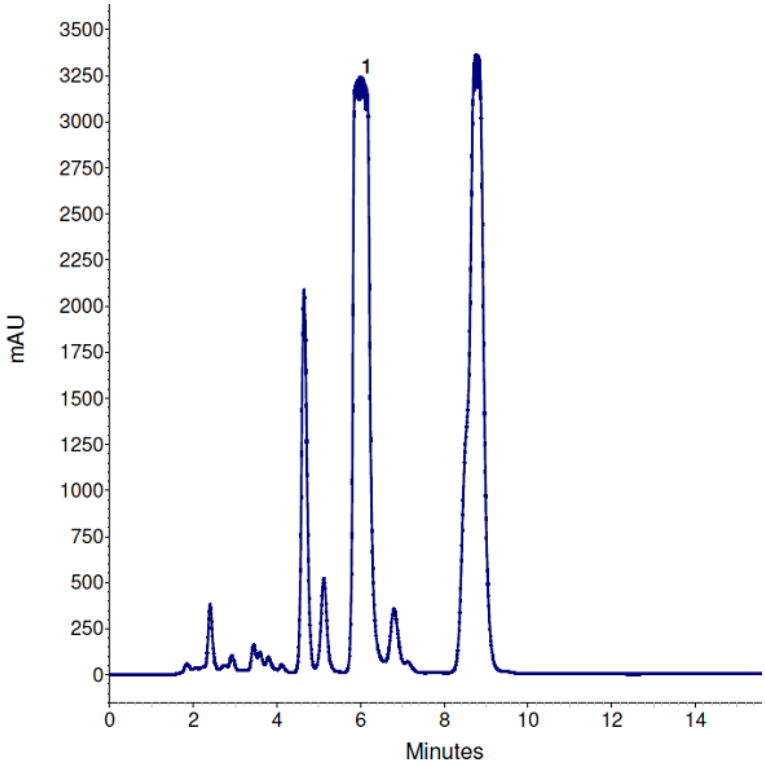


Fig. 2

Analytical chromatogram of the pepper extract (injection volume 2 µl) before purification

Column: Eurospher II 100-5 C18, 250 x 4 mm ID

Once satisfying analytical results are made, the user is able to transfer the method easily to the preparative scale. This means that the system, column and method parameters have to be scaled up. Before transfer, the injection volume was scaled up in the analytical mode. The resulting chromatogram with 40 µl injection volume and therewith a column overload can be seen in figure 3.



1 Piperine

Fig. 3

Analytical chromatogram of the pepper extract with column overload (40 µl injected) before purification used to scale up to the preparative mode

Column: Eurospher II 100-5 C18, 250 x 4 mm ID with integrated precolumn

Now the AZURA preparative HPLC system and a Eurospher II C18 phase filled in the KNAUER Vertex Plus AX hardware with axial compression were used. The resulting preparative chromatogram can be seen in fig. 4. The marks indicate when the Piperine fraction was collected to reach the highest purity.

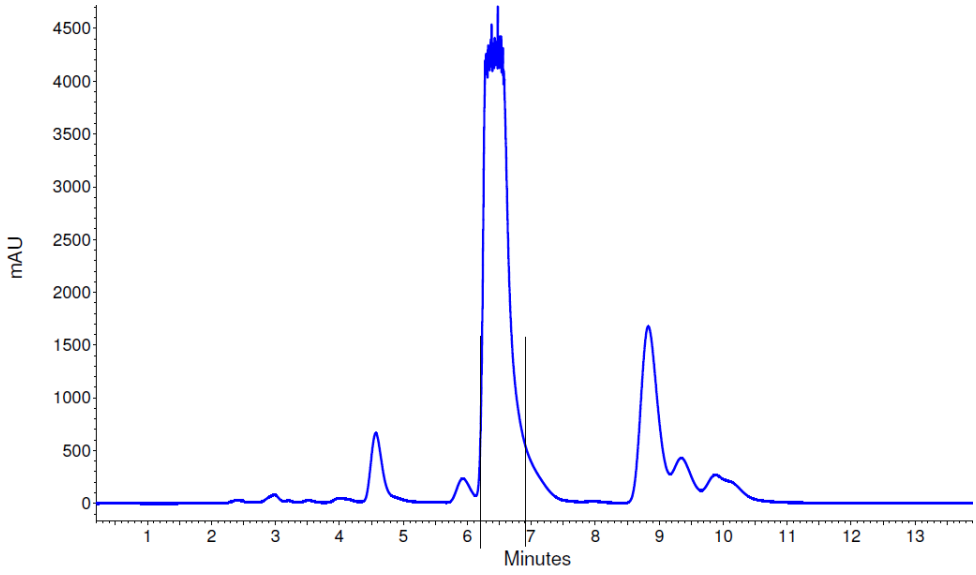


Fig. 4

Preparative chromatogram of the pepper extract for purification of Piperine

Column: Eurospher II 100-5 C18, 250 x 20 mm ID with precolumn 30 x 20 mm

The Piperine fraction was collected using a switching valve. To control the purity of this fraction, the next step was a high resolving HPLC method using the BlueShell classic core shell column and the AZURA compact system. The highest resolution is needed in this step, because all impurities have to be separated from the target peak Piperine to discover the purity of the target substance. The chromatogram of the Piperine fraction´s purity control can be seen in figure 5.

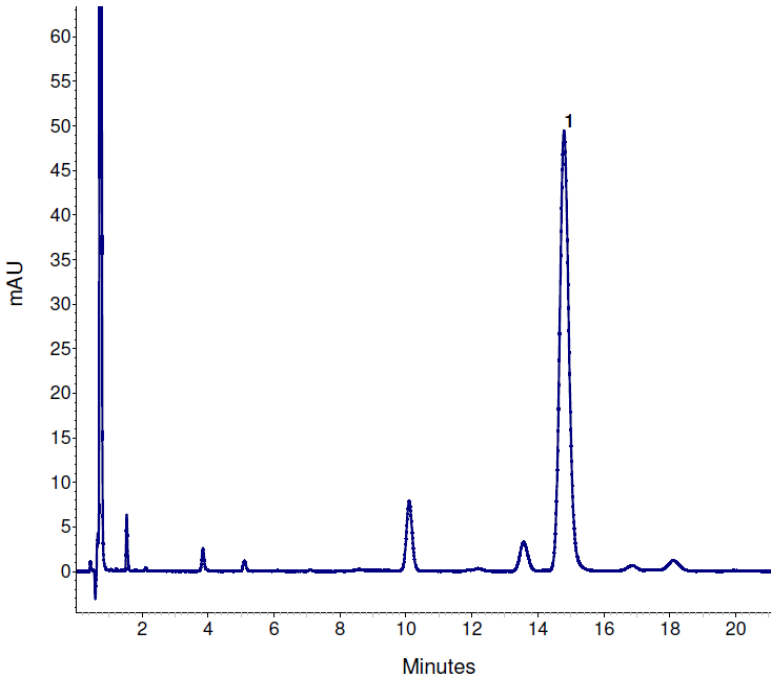


Fig. 5

Analytical chromatogram of the Piperine fraction after purification by preparative HPLC

Column: BlueShell 80-4.5 classic C8, 150 x 3 mm ID

To get a comparison, fig. 6 shows the overlay of the chromatograms measured before (green) and after (blue) purification by preparative HPLC. It becomes obvious that the extract could be purified in a high range via preparative HPLC. Retention times vary because different column selectivities and different method parameters were used.

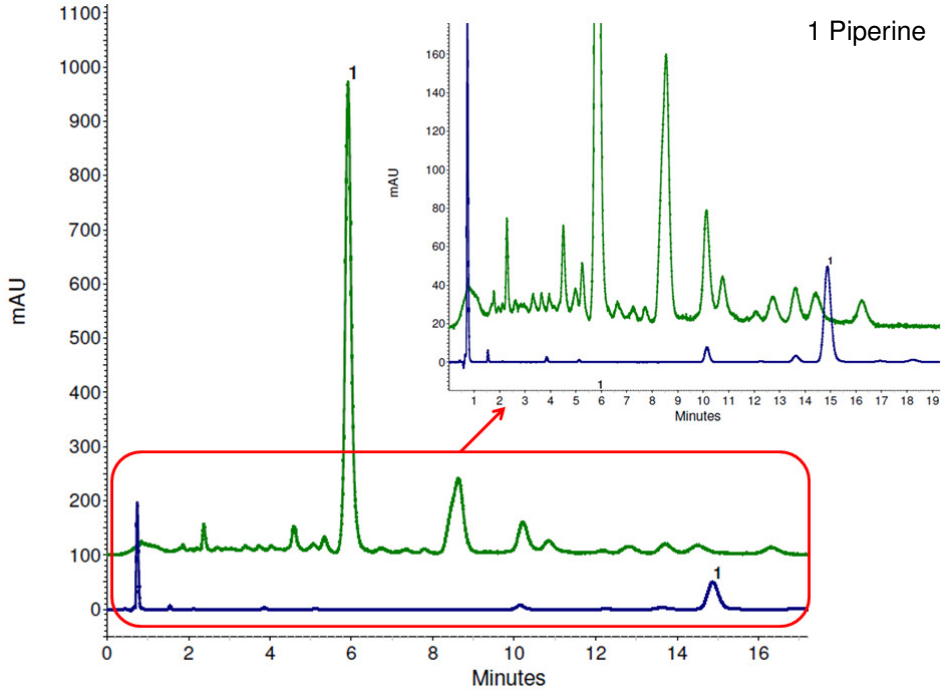


Fig. 6

Analytical chromatograms recorded at 210 nm before (green) and after (blue) purification by preparative HPLC

Green: Column: Eurospher II 100-5 C18, 250 x 4 mm ID

Blue: Column: BlueShell 80-4.5 classic C8, 150 x 3 mm ID

The BlueShell classic phase with a C8 modification was chosen, because it gave the best separation and therewith the best purity control of Piperine. With the first tested BlueShell classic C18 column there was still an impurity eluting at the same time as Piperine. With the C8 modification, retention was much better for Piperine what can easily be seen from figure 6. Piperine elutes comparatively late after about 15 min and is completely separated

from all impurities. As a result, in this case the purity control was done on a column with different selectivity as the one used for purification.

To get an exact idea how good the purification step works, the peak area of Piperine can be determined and compared to the overall peak areas of the impurities. For this method, the resulting purity of Piperine was greater than 81 % regarding the peak area. For this calculation, the injection peak resulting from acetone was not regarded, because acetone can easily be evaporated after purification.

Conclusion

The AZURA line is perfectly suited for all the analyses occurring during preparative method development. With AZURA compact, the user can easily run analyses in the analytical scale to screen for columns that may be applied in the preparative scale. Via a scale up to the AZURA Preparative HPLC system, purification of Piperine can be done with one easy run. The combinable AZURA elements help the user to set up a system that is perfectly suited for the individual separation task.

After the purification step via preparative HPLC and fractionation of the target peak, a purity control can easily be done applying the same AZURA compact system as used before during method development. The system is able to run a sensitive and fast analysis of the purified Piperine fraction. Applying a BlueShell classic C8 column leads to very sharp peaks and therewith to very good resolution and low detection limits.

References

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Physical properties of recommended analytical screening column

Based on an ultra-pure silica gel, Eurospher II is a high performance column material for analytical, semi preparative and process-scale applications. Eurospher II features very narrow particle and pore size distributions, as well as outstanding mechanical stability. Eurospher II silica gel is perfectly suited to take on routine analyses as well as the most ambitious chromatography tasks.

Stationary phase	Eurospher II 100-5 C18
USP code	L1
Pore size	100 Å
Pore volume	0.8 ml/g
Specific surface area	320 m²/g
Particle size	5 µm
Form	spherical
% C	15 %
Endcapping	yes
Dimensions	250 x 4 mm Vertex Plus with integrated precolumn
Order number	25WE181E2J

Physical properties of recommended preparative column

Based on an ultra-pure silica gel, Eurospher II is a high performance column material for analytical, semi preparative and process-scale applications. Eurospher II features very narrow particle and pore size distributions, as well as outstanding mechanical stability. Eurospher II silica gel is perfectly suited to take on routine analyses as well as the most ambitious chromatography tasks.

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Pore volume	0.8 ml/g
Specific surface area	320 m²/g
Particle size	5 µm
Form	spherical
% C	15 %
Endcapping	yes
Dimensions	250 x 20 mm Vertex Plus AX with precolumn
Order number	03PE181E2J and 25PE181E2J

Physical properties of recommended analytical column for purity control

To obtain ultra-high performance results without the disadvantage of high backpressure, our new BlueShell classic columns are your first choice. BlueShell classic columns are packed with special 4.5 µm core-shell particles, developed to provide improved speed, higher resolution, and reduced eluent consumption, all while keeping moderate HPLC backpressures.

Stationary phase	BlueShell classic 80-4.5 C8 core shell
USP code	L1
Pore size	80 Å
Pore volume	0.8 ml/g
Specific surface area	130 m²/g
Particle size	4.5 µm
Form	core shell
% C	4 %
Endcapping	yes
Dimensions	150 x 3 mm
Order number	15CD081SHI

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Recommended Instrumentation

The AZURA line has been designed to provide highly adaptable HPLC solutions with up to date features. AZURA will offer you reliable technology, extensible equipment, and flexibility in your choice of applications, making the instruments a future-proof investment. The system is freely customizable. The detector can be chosen from the wide range of KNAUER detectors. If you need more flexibility, use the ASM 2.1L elements to put together your own system according to your needs!

Analytical System: AZURA Compact

The Compact HPLC HPG consists of an ASM 2.1L-based high pressure gradient pump element with integrated 2-channel degasser. The system is completed by a UVD 2.1L, (a UV/Vis detector with one variable wavelength) and comes with an eluent tray for safe storage of up to 6 bottles.

Description	Order No.
ASM 2.1L with 2 channel degasser, HPG pump unit with mixing chamber	AYBMXXIA
AZURA UVD 2.1L: 190-750 nm, variable single wavelength UV/VIS detector	ADA01
Analytical flow cell UV, 10 mm path length, 10 µl volume, 1.1 mm ID, 1/16", stainless steel	A4061
Autosampler KNAUER Optimas (analytical HPLC autosampler standard)	A5007
AZURA Eluent tray E 2.1L for up to 6 x 1000 ml bottles	AZC00
Magnetic clip for analytical column OD ¼"	A9847
PC with Chromatography software:	Upon request

Preparative System: AZURA Prep

The AZURA Preparative HPLC system is a complete preparative low pressure gradient HPLC system for purification of milligram and gram samples. It consists of a preparative HPLC pump with LPG valve block, a UV/VIS detector and a customizable combination module. The pump P 2.1L with LPG valve block enables to form dynamically eluent mixtures from up to 3 different eluents, or to run a binary gradient.

Description	Order No.
AZURA ASM 2.1L for Preparative HPLC with 12 port multi position valve, 1/8" connectors, 6 port 3 channel injection valve, 1/8" connectors, Pump without pressure sensor, 50 ml pump head	AYFAEEAD
AZURA UVD 2.1L: 190-750 nm, variable single wavelength UV/VIS detector	ADA01
Preparative flow cell UV	A4066
AUURA P 2.1L: Preparative HPLC pump with 250 ml/min pump head, stainless steel	APE20LA
AZURA Eluent tray E 2.1L for up to 6 x 1000 ml bottles	AZC00
AZURA LPG module for Pump P 2.1L: Ternary LPG module for flow rates up to 220 ml/min, stainless steel	AZZ00AB
Dynamic mixing chamber for preparative HPLC gradient systems, stainless steel	A0581
PC with Chromatography software:	Upon request

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