

No blood(y) stress - Phenolic antioxidants in olive oil and olive oil wastewater

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SUMMARY

The content of polyphenols, or biophenols, in olive oil is interesting due to their attributed benefits and impact on human health. Only with a certain amount of biophenols the manufacturer can advertise the product to have a positive effect on health¹. But also, the wastewater incurring in olive oil production can still contain the coveted polyphenols. These wastewaters, which are mostly discarded, might be a source for a possible recovery of these biologically interesting constituents².

INTRODUCTION

Polyphenols are secondary plant compounds that can offer various health benefits. They can act as antioxidants or be helpful due to their anti-inflammatory effects, for example. There are many foods, plants and vegetables which are rich in polyphenols but in this application the focus was set on olive oil and wastewater from olive oil production. With Commission Regulation (EU) No 432/2012 of 16 May 2012 a list of permitted health claims made on food was established³. Referring to this list, olive oils can be labeled with the addition "*Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress*". The claim may be used only for olive oil which contains at least 5 mg of hydroxytyrosol and its derivatives (e.g.

oleuropein complex and tyrosol) per 20 g of olive oil. In order to bear the claim information shall be given to the consumer that the beneficial effect is obtained with a daily intake of 20 g of olive oil³. The profile of biophenolic compounds in olive oils and its wastewater is quite complex with far over 20 possible substances. A detailed method for profiling olive oil samples is provided by the International Olive Council⁴. Most of the used standards described in that method are commercially unavailable, thus in the following application the determination of only eight compounds (3-hydroxytyrosol, tyrosol, 4-hydroxyphenylacetic acid, vanillic acid, caffeic acid, syringic acid, oleuropein, and quercetin) was conducted with an adapted method.

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RESULTS

For peak identification, single standards of the eight phenolic compounds were measured. Thereafter a mixed standard stock solution was prepared. For calibration, the stock solution was diluted to five different levels (**Tab. 1**).

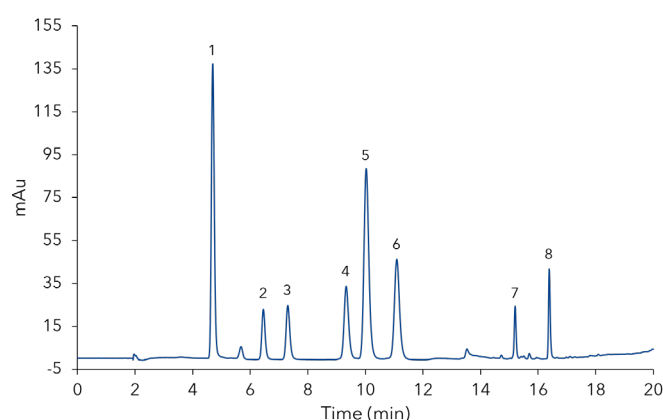


Fig. 1 Chromatogram of mixed standard at calibration level 5.
1 - 3-hydroxytyrosol, 2 - Tyrosol, 3 - 4-hydroxyphenylacetic acid, 4 - Vanillic acid, 5 - Caffeic acid, 6 - Syringic acid, 7 - Oleuropein, 8 - Quercetin.

Tab. 1 Concentrations of calibration levels

Substance	Level 1 (µg/ml)	Level 2 (µg/ml)	Level 3 (µg/ml)	Level 4 (µg/ml)	Level 5 (µg/ml)
3-Hydroxytyrosol	4.400	6.600	13.200	66.000	132.000
Tyrosol	1.233	1.850	3.700	18.500	37.000
4-Hydroxyphenylacetic acid	1.967	2.950	5.900	29.500	59.000
Vanillic acid	0.867	1.300	2.600	13.000	26.000
Caffeic acid	1.367	2.050	4.100	20.500	41.000
Syringic acid	0.867	1.300	2.600	13.000	26.000
Oleuropein	1.967	2.950	5.900	29.500	59.000
Quercetin	0.733	1.100	2.200	11.000	22.000

The calibration was successful with a linearity of $R^2 \geq 0.9996$ for all compounds. Based on the calibration measurements the limit of detection (LOD) and limit of quantification (LOQ) were calculated (**Tab. 2**). For LOD a signal-to-noise ratio (S/N) of $S/N=3$ was taken as basis of calculation and for LOQ a ratio of $S/N=10$ was specified.

Tab. 2 Calculated LOD and LOQ

Substance	LOD $S/N=3$ (µg/ml)	LOQ $S/N=10$ (µg/ml)
3-Hydroxytyrosol	0.794	2.645
Tyrosol	1.316	4.386
4-Hydroxyphenylacetic acid	1.944	6.481
Vanillic acid	0.641	2.138
Caffeic acid	0.382	1.274
Syringic acid	0.463	1.543
Oleuropein	1.963	6.542
Quercetin	0.431	1.437

Five commercially available olive oils were tested and prepared according to the Internal Olive Council sample preparation method (section 5.1)⁴ with the changes mentioned in the sample preparation section. All samples were marked as "native extra" but none of the samples was labelled according to Health Claims Regulation. Exemplarily two chromatograms of olive oil samples are shown. Three of the calibrated substances were detected in the sample (**Fig. 2**). Except for 3-hydroxytyrosol the calculated amounts were below the LOQ of the method.

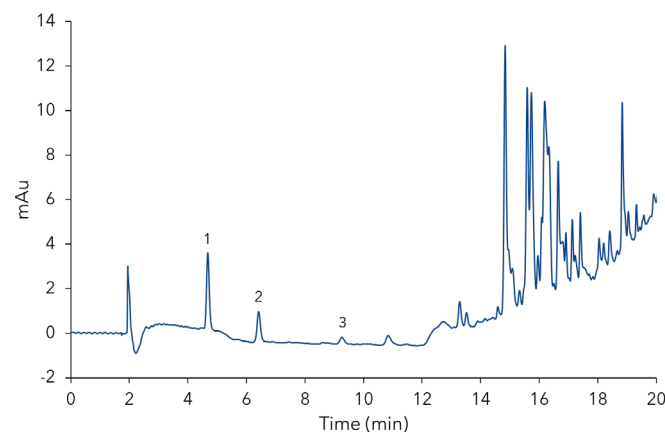


Fig. 2 Chromatogram of olive oil sample 2. 1 - 3-hydroxytyrosol, 2 - Tyrosol, 3 - vanillic acid.

RESULTS

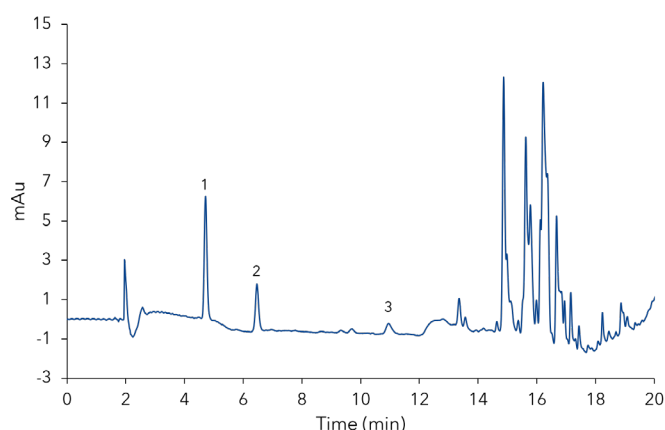


Fig. 4 Chromatogram of olive oil sample 3. 1 - 3-hydroxytyrosol, 2 - Tyrosol, 3 - Syringic acid.

In the second sample also some of the calibrated compounds were detected. Again except for 3-hydroxytyrosol the amounts were below the calculated limit of quantification or even limit of detection. The detectable substances were quantified (Tab. 3, Fig. 5). Caffeic acid and 4-hydroxyphenylacetic acid were neither found in olive oil nor in wastewater samples.

Tab. 3 Results of sample measurement, * = below LOQ/LOD

Substance	Sample 1 (µg/ml)	Sample 2 (µg/ml)	Sample 3 (µg/ml)	Sample 4 (µg/ml)	Sample 5 (µg/ml)
3-Hydroxytyrosol	4.107	3.641	6.375	3.433	4.289
Tyrosol	2.620*	2.594*	4.107*	2.813*	3.352*
Vanillic acid	-	0.393*	-	-	-
Syringic acid	0.322*	-	0.386*	-	0.207*
Oleuropein	1.375*	-	-	-	1.859*
Quercetin	1.131*	-	-	1.430*	1.623

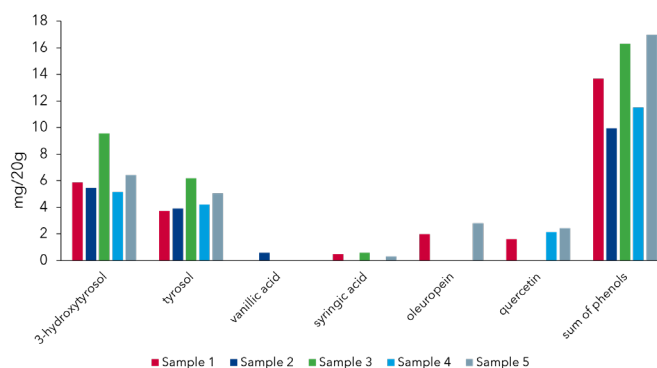


Fig. 3 Amounts of phenols in samples in mg/20g.

Tyrosol and 3-hydroxytyrosol were found in each of the five samples whereas the other calibrated compounds show different composition patterns. Instead of using an internal standard, the recovery rate was determined. Therefore, one of the samples was spiked with the mixed standard at different concentrations and extracted according to sample preparation procedure.

Tab. 4 Recovery rate for extraction procedure

Substance	Recovery rate (%)
3-Hydroxytyrosol	99.77
Tyrosol	92.28
4-Hydroxyphenylacetic acid	88.86
Vanillic acid	98.96
Caffeic acid	92.20
Syringic acid	90.25
Oleuropein	52.93
Quercetin	73.77

Furthermore, two wastewater samples from an olive oil production process were measured. The target was to verify if the wastewater still contains amounts of polyphenols which could be of interest for recovery and/or re-use. Recovery rates for the wastewater samples were not determined.

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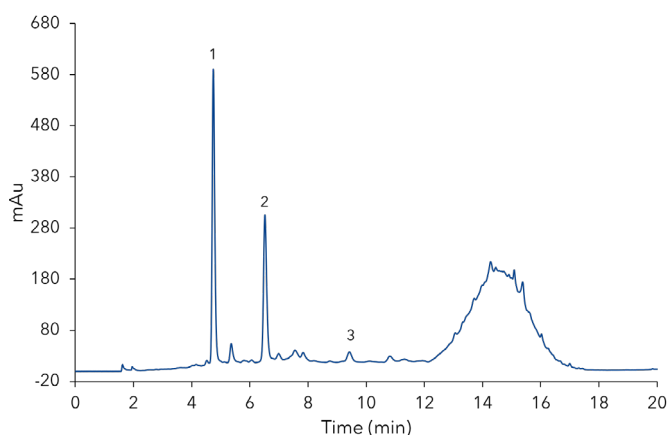


Fig. 5 Chromatogram of olive oil wastewater sample 1. 1 - 3-hydroxytyrosol, 2 - Tyrosol, 3 - Vanillic acid.

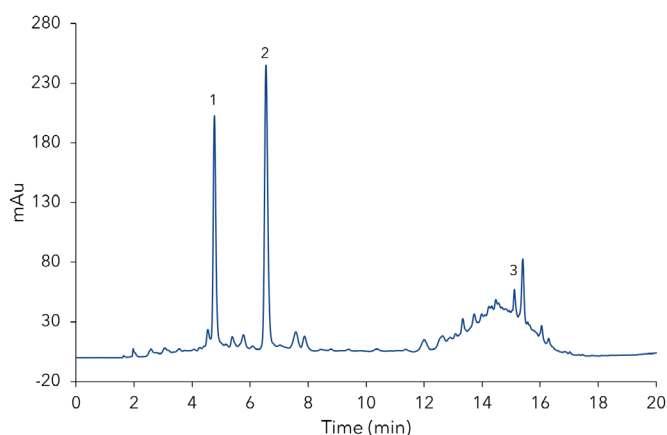


Fig. 6 Chromatogram of olive oil wastewater sample 2. 1 - 3-hydroxytyrosol, 2 - Tyrosol, 3 - Oleuropein.

Both wastewater samples contain high residues of 3-hydroxytyrosol and tyrosol as well as small amounts of other of the calibrated substances (**Tab. 5**).

Tab. 5 Recovery rate for extraction procedure

Substance	Wastewater 1 (µg/ml)	Wastewater 2 (µg/ml)
3-Hydroxytyrosol	537.336	179.115
Tyrosol	484.373	413.389
4-Hydroxyphenylacetic acid	-	-
Vanillic acid	16.258	-
Caffeic acid	-	-
Syringic acid	-	-
Oleuropein	-	48.740
Quercetin	-	-

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SAMPLE PREPARATIONS

Single standards were dissolved in methanol. Out of these single standards a mixed standard was prepared. Olive oil samples were prepared according to International Olive Council method (section 5.1 Sample preparation)⁴, but without the use of an internal standard. Furthermore, the 0.45 µm PVDF (polyvinylidene fluoride) filter was replaced by a 0.45 µm RC (regenerated cellulose) filter. Wastewater samples were prepared with manual solid phase extraction using CHROMABOND C18, 45 µm, 3 ml/500 mg SPE columns⁵. The applied SPE method was taken from the Macherey Nagel application database⁶.

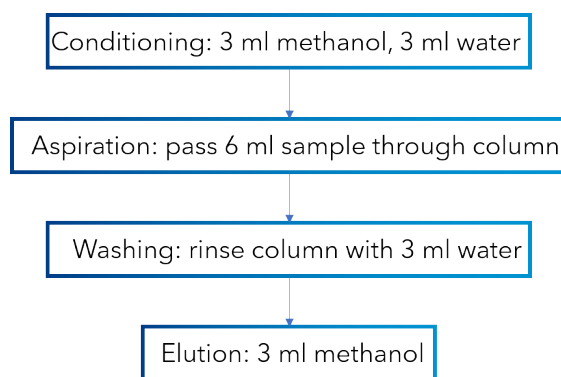


Fig. 7 SPE method parameters

CONCLUSION

Although the measured olive oil samples were not labelled with the claim "*Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress*"³, the samples would meet the requirements. For all extracts an amount of at least 5 mg of 3-hydroxytyrosol and its derivatives (e.g. oleuropein complex and tyrosol) per 20 g of olive oil were

determined (Tab. 3, Fig. 4). The analyzed wastewater samples showed high amounts of 3-hydroxytyrosol and tyrosol (Tab. 5). With an appropriate sample preparation and possibly preparative HPLC purification, it should be feasible to recover these substances and make them usable for other areas of application.

REFERENCES

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MATERIALS AND METHODS

Tab. 6 Method settings

Column temperature	30 °C
Injection volume	10 µl
Injection mode	Partial loop
Detection	UV 280 nm
Data rate	20 Hz
Time constant	0.05 s

Tab. 7 Pump parameters

Eluent (A)	0.2% phosphoric acid		
Eluent (B)	methanol		
Flow rate	1.2 ml/min		
Gradient	Time (min)	% A	% B
	0	90	10
	3	80	20
	10	75	25
	18	0	100
	20	0	100
	20.02	90	10
	23	90	10

Tab. 8 System configuration

Instrument	Description	Article No.
Pump 1	AZURA P6.1L HPG, 10 ml	APH35EA
Autosampler	AZURA AS 6.1L	AAA00AA
Detector	AZURA DAD 6.1L	ADC11
Flow cell	Analytical KNAUER pressure-proof UV flow cell cartridge	AMC38
Thermostat	AZURA CT 2.1	ATC00
Column	Eurospher II 100-5 C18A, 250 x 4 mm ID with precolumn	25WE184E2J
Software	ClarityChrom 8.2.3 - Workstation, autosampler control included	A1670
Software	ClarityChrom 8.2.3 - PDA extension	A1676

