ANALYSIS OF HYDROXYLATED CYTOKININ DERIVATIVES

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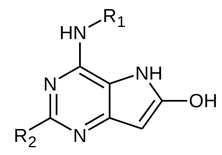
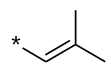
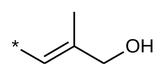
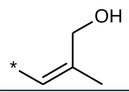
Introduction

Cytokinins, a group of plant hormones, have crucial physiological impact on the plant growth and development. Their derivatives hydroxylated on the purine moiety (OHCK; Tab. 1) has been previously synthesized by *in vitro* reaction with xanthine:NAD+ oxidoreductase (XDH). The enzyme is involved in purine metabolism with primary function to catalyze oxidation of hypoxanthine via xanthine to uric acid (Fig. 1). In order to determine whether OHCK occur naturally in plants, purification procedure based on solid phase extraction and UHPLC-MS/MS methods for their qualitative and quantitative analysis have been developed. Plant material for analysis was selected according to literature and monitoring of XDH activity in plant tissue extracts by native PAGE.

Tab. 1 – Structures of OHCK



Substance	Abbrev.	R_1	R_2
8-hydroxyisopentenyladenin	8-OHiP		-H
2,8-dihydroxyisopentenyladenin	2,8-OHiP		-OH
8-hydroxy-*trans*-zeatin	8-OHtZ		-H
2,8-dihydroxy-*trans*-zeatin	2,8-OHtZ		-OH
8-hydroxy-*cis*-zeatin	8-OHcZ		-H
2,8-dihydroxy-*cis*-zeatin	2,8-OHcZ		-OH

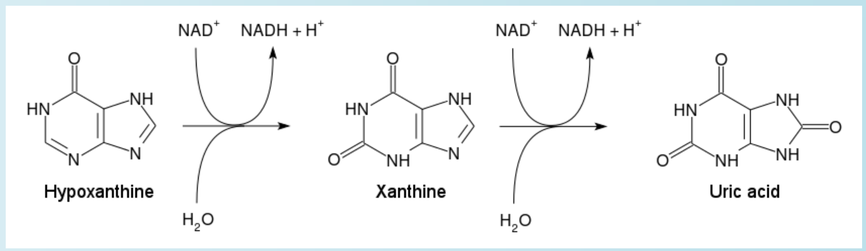


Fig. 1 – Purine catabolism by XDH

Materials and methods

- Different developmental stages and tissues of *Arabidopsis thaliana*, *Pisum sativum* and *Hordeum vulgare* were used as plant material.
- MS (Waters XEVO TQ) has been tuned for the best sensitivity using OHCK standards and collision spectra were acquired. Analysis were performed in MRM mode.
- UHPLC (Waters ACQUITY) separation was performed on column BEH C8 (150 x 2.1 mm; 1.7 µm) by gradient elution – methanol /15 mM ammonium formate.
- Strata-X and Oasis MCX columns were applied for OHCK purification. Recoveries were determined using plant extract as background.
- XDH activity in the plant tissue extracts was monitored by activity staining with formazan dyes after native electrophoresis.
- Selected plant material was analyzed by UHPLC-MS/MS; [$^2H_5$]-*trans*-zeatin-9-glucoside was used as internal standard.

Results

- Fig. 2 represents example of OHCK collision spectrum. Three main obtained fragments are shown on the structural formula of parental 2,8-OHiP.
- Baseline separation of both pairs of isomers was achieved (Fig. 3).
- Retention times for each studied OHCK as well as some analytical parameters are summarized in Tab. 2. Detection limits were in range of 40-70 fmol for monohydroxy and 180-450 pmol for dihydroxy derivatives.
- Response of MS detector for each OHCK was linear ranging from its respective detection limit to 10 pmol. Higher amounts of standards were not measured.

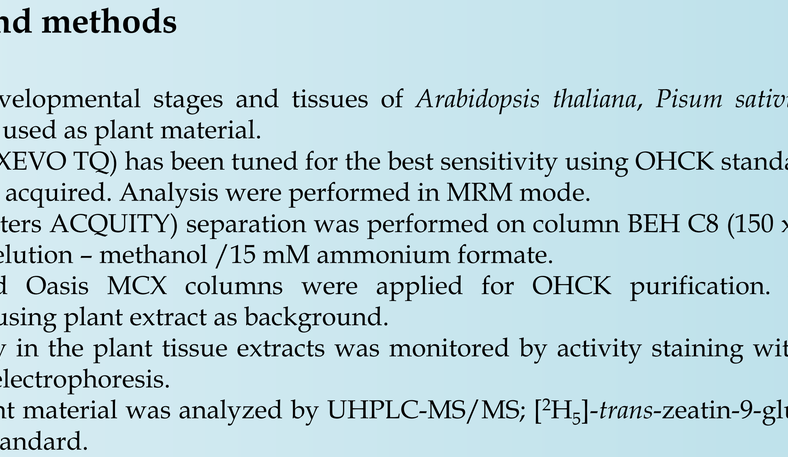


Fig. 2 – Collision spectrum of 2,8-OHiP

Tab. 2 – Analytical parameters of UHPLC-MS/MS method

	Retention time [min]	LOD [pmol]	LOQ [pmol]	LDR[a] [pmol]	Equation	R^2
2,8-OHtZ	3.30	0.45	1.00	0.45-10	y = 49.98x – 13.04	0.9974
2,8-OHcZ	4.02	0.18	0.43	0.18-10	y = 181.3x + 1.13	0.9999
2,8-OHiP	6.79	0.36	0.9	0.36-10	y = 141.9x – 37.01	0.9869
8-OHtZ	6.58	0.04	0.08	0.02-10	y = 434.7x – 6.5	0.9912
8-OHcZ	6.85	0.05	0.1	0.04-10	y = 1106x – 30.8	0.9998
8-OHiP	9.45	0.07	0.12	0.07-10	y = 490.9x – 8.58	0.9995

[a] linear dynamic range; concentrations over 10 pmol were not measured

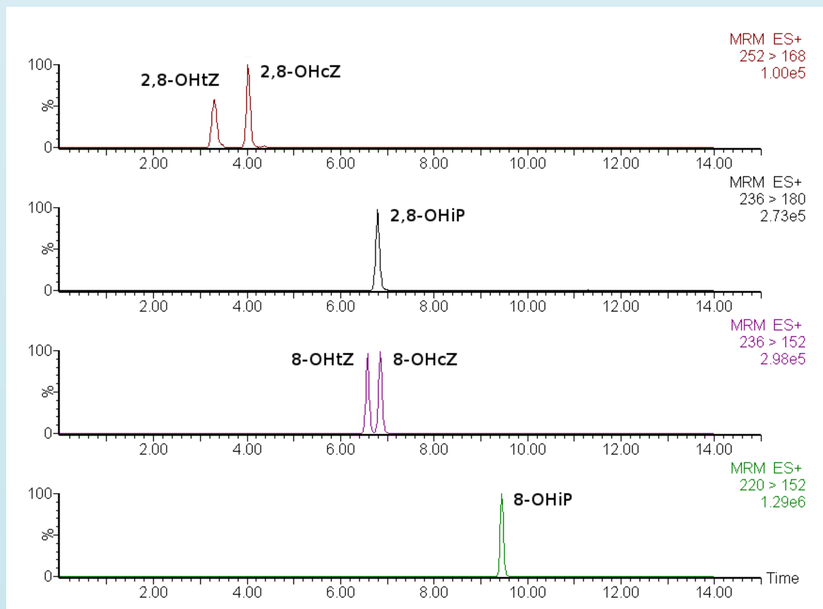


Fig. 3 – Chromatogram of OHCK standards

- Strata-X, a reverse phase sorbent, was unable to effectively retain more polar dihydroxy OHCK. Recoveries for those substances were 52 % at the best (Tab. 3).
- As mixed-mode sorbent, MCX retained analytes either by cation exchange and/or by hydrophobic interaction. Recoveries close to 100 % were achieved using solution of ammonium hydroxide in methanol as eluent.

Tab. 3 – Recoveries of solid phase purification of OHCK

			Recovery [%]					
SPE	**Rep.**	**Elution**	**2,8-OHtZ**	**2,8-OHcZ**	**8-OHtZ**	**8-OHcZ**	**2,8-OHiP**	**8-OHiP**
Strata-X	# 1	MeOH	32.5	/[a]	/	/	/	75.9
	# 2	MeOH	30.2	52.2	60.0	51.3	28.0	50.2
Oasis MCX	# 1	E1[b]	111.3	/	/	/	/	0.0
	# 1	E2[b]	3.4	/	/	/	/	113.4
	# 2	E2	102.9	100.7	111.7	98.4	84.0	102.5
	# 3	E2	121.4	71.0	99.8	115.7	79.3	52.4

[a] not applied
[b] E1 = 0.35 M ammonium hydroxide; E2 = 0,35 M ammonium hydroxide in 60% methanol

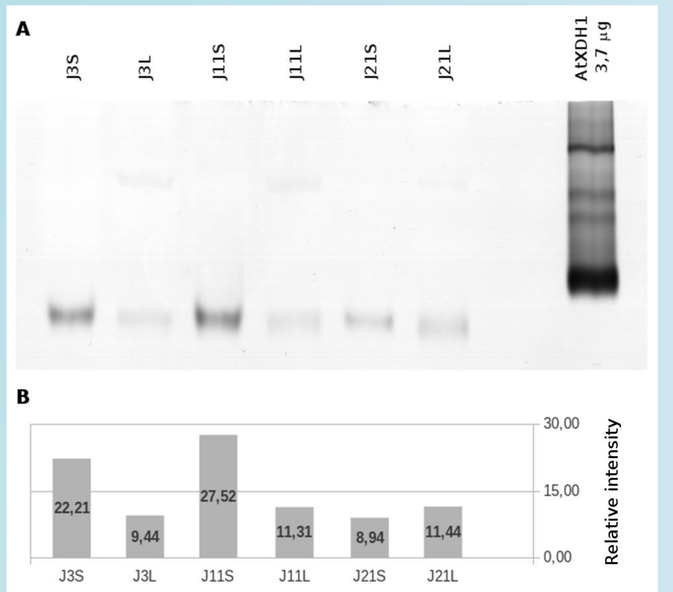


Fig. 4 – Electropherogram of XDH from barley (A) and graph of relative intensity of bands (B).
Abbreviations of samples consist of letter J (stands for barley), age in weeks and letters S or L (stem or leaf).

- Activities of XDH in different tissues were compered after staining as shown on Fig. 4 - A. Recombinant XDH from *Arabidopsis* was used as standard.
- Relative intensities representing XDH activities of individual samples (Fig. 4 – B) were calculated after image analysis considering AtXDH1 as 100 %.
- The most intensive band (beside AtXDH1) represents sample of 11 weeks old stem of barley (J11S).
- Samples selected this way has been used for OHCK analysis.

- Signal corresponding to 8-OHtZ has been detected only in senescent leaves of pea, Fig. 5 – B.
- Due to low intensity of signal, collision spectrum could not be acquired for further confirmation of its identity.

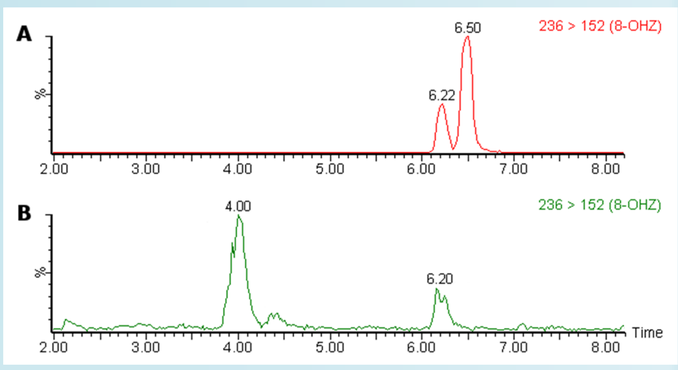


Fig. 5 – Chromatogram of standards (A) and sample from senescent pea leaves (B)

Conclusion

Procedure for purification of OHCK from plant material and their subsequent analysis has been developed and some of its analytical parameters determined. Samples for analysis were chosen based on the literature and comparison of XDH activities in various plant tissues and developmental stages. Signal possibly belonging to 8-OHtZ has been detected in the sample from senescent pea leaves, however its identity could not be verified. Experiment will be repeated with larger amount of initial plant material to compensate potentialy low levels of OHCK in the sample.

References

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Acknowledgement

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