

Extraction of Bitter Acids from Hops and Hop Products Using Pressurized Solvent Extraction (PSE)

Jiří Čulík^{1,3}, Marie Jurková¹, Tomáš Horák¹, Pavel Čejka¹, Vladimír Kellner¹,
Josef Dvořák¹, Pavel Karásek² and Michal Roth²

ABSTRACT

J. Inst. Brew. 115(3), 220–225, 2009

The EBC method 7.7, currently used for determination of bitter acids in hop products, is a time-consuming and laborious extraction technique. In this paper, our aim was to propose a new extraction method based on Pressurized Solvent Extraction (PSE) sometimes also called Pressurized Fluid Extraction (PFE) or Accelerated Solvent Extraction (ASE). Compared to conventional extractions, PSE offers a number of important benefits. PSE on OnePSE® automated extractor was used for extraction of α - and β -acids from hops and hop products and the parameters influencing extraction efficiency and the influence of the sample preparation method were studied. The quantitative determination of α - and β -acids in the extracts was accomplished by using an HPLC apparatus equipped with diode array detector. The experimental results were compared with those obtained by the standard EBC 7.7 method and the two methods were found to be fully compatible

Key words: analysis, bitter acids, hops, hop products, pressurized solvent extraction, PSE.

INTRODUCTION

Hops, one of the three main brewery raw materials, provide beer, apart from other technologically important properties, with its typical bitter flavour and aroma. In view of the relatively high price of hops and hop products (granules, hop extract) the growers and suppliers are interested in determining the content of compounds affording the bitter flavour with maximum precision and accuracy.

In terms of commercial value and brewery use, hops varieties or cultivars can be divided into mild or aromatic ones with a pleasant hop aroma but a lower content of bitter acids, with the typical representative (Saaz semi-early red bines hops) grown in the Czech Republic, and the bitter or high-bitter acid varieties with a less pleasant

aroma (varieties cultivated mostly in the USA, Great Britain and Australia). Increased attention is currently being paid to varieties obtained by crossing the two above sorts, thus combining the advantages of the higher content of bitter acids and of hop volatiles. For example, in this country they are represented by the varieties Bor and Premiant. They are usually denoted semi-aroma or dual-purpose hops. Hop bitter acids comprise α - and β -acids. Bitter α -acids are composed of three main components: n-humulone, ad-humulone and co-humulone. Likewise, bitter β -acids consist of n-lupulone, ad-lupulone and colupulone. Varieties of the Saaz hops and similar Bavarian varieties (Hallertauer, Tettnang, and Hersbrucker) are also characterized by a lower proportion of co-humulone and a higher proportion of β -acids. The content of α -acids is 4 to 5% and β -acids amount to 2 to 5%. The content of bitter α -acids in the dual-purpose varieties is up to 8%, whereas in high-alpha varieties it can reach 12%. Some varieties that have been cultivated feature α -bitter acid levels in excess of 15% and these have been termed “super-alpha” varieties and are especially suited for the production of hop extracts.

More important for brewery production are α -acids, which are only marginally soluble in water. Their brewing isomerization by boiling in a weakly acid environment yields iso- α -acids, which are more soluble and exhibit considerable organoleptic bitterness. The β -acids are less important for brewery production because additional hydrophobic chains in their structure make them even less soluble in water. The conversion of β -acids to the corresponding isomerized products during wort boiling is therefore very low.

In Europe, the content of α -acids in hops and hop products has traditionally been determined by the wide-spread conductometric method^{1,6,12} based on the precipitation of bitter acids with lead salt. This method yields a conductometric value (CV) in percent. Today, the method of choice for determining α - and β -acids is high-performance liquid chromatography (HPLC), which provides more realistic and exact data. HPLC can be used to determine only α - and β -acids^{2,13}, or, at the same time, their isomerized products³. The method can be used also for determining iso- α -acids and reduced iso- α -acids in hop products⁴.

The common drawback of conductometric and HPLC methods is the laborious and time-consuming procedure of sample preparation.

¹Research Institute of Brewing and Malting, PLC, Brewing Institute Prague, Lípová 15, CZ-120 44 Prague 2, Czech Republic.

²Institute of Analytical Chemistry, Academy of Sciences of the Czech Republic, Veveří 97, CZ-611 42 Brno, Czech Republic.

³Corresponding author. Email: culik@beerresearch.cz.

Effort has therefore been made to find other more suitable methods. Among the state-of-the-art extraction methods used for extracting bitter compounds from hops, the most widespread is supercritical fluid extraction with carbon dioxide^{8-11,14}. Another modern method, pressurized fluid extraction (PFE), has so far been employed for extracting different representatives of polyphenols^{7,15-17}. Our aim was to develop a less laborious and reliable procedure for extraction of bitter acids using pressurized solvent extraction (PSE).

MATERIALS AND METHODS

Reagents, solutions and samples

The individual hop extract standard, namely, calibration standard ICE 2, was obtained from Labor Veritas, Zurich. The measurements of calibration curves and experiments to optimise HPLC analysis were carried out with methanol, water and ortho-phosphoric acid obtained from Riedel-de Haën, Germany, HPLC grade.

The experiments aimed at PSE extraction were carried out with methanol, diethylether, ethanol, propanol, and dichloromethane as extraction media. All solvents were obtained from Sigma-Aldrich, Czech Republic and were analytical grade. Prior to extraction the samples were mixed with glass beads (Balotina 7, 570–700 μm , GlassDekorService, Czech Republic), diatomaceous earth (Speed matrix, Applied Separations), river sand (Ottawa sand, Applied Separations), and sea sand (KORFU-Sidari and Sigma-Aldrich).

Samples, hops, and hop pellets and extracts were collected and prepared in the Czech Republic during the crop years 2006–2008.

PSE instrumentation

The OnePSE extractor (Applied Separations, USA) (Fig. 1) is able to operate in two modes. The first is the

classical PFE extraction mode described in EPA 3545⁵ in detail (mode A – static mode). The second mode was developed especially for the analytes with high affinity to resorption into the matrix during the depressurization phase (mode B – semidynamic mode).

Static mode

The extraction step starts by heating the extractor to the selected temperature. The prepared samples of hops, ground (and if needed homogenised) and mixed with inert material, are weighed and put directly into the extraction cartridge. Then, the extraction cartridge is inserted into the extraction device and the whole system is immediately purged with nitrogen to remove residual air to prevent oxidation of analytes.

In the next step, the whole system is pressurized to half the required value and held in the preheating period (typically 2 min) to allow proper heating of the vessel, sample and solvent. Then the system is pressurized to the final value and the static period (typically 5–30 min) starts. At the end of the static period, the system is completely discharged to the collecting vial (1 min). In the case of series of several static cycles (typically 2–5), the system is pressurized again, preheated (1 min), and the next static cycle is carried out. After the last static period and discharging of the extract to the vial, the device is flushed, at first by small amounts of solvents and finally by a stream of nitrogen to remove the residual solvents from the system.

Semidynamic mode

In this mode, the initial phase of the process is the same as in the static mode. The difference occurs at the end of the static period. In semidynamic mode, only 5–10% of the extract is discharged from the vessel, and the system is immediately repressurized with the neat solvent to the set value. The time of the static period is typically 10–60 sec, and the static period is repeated 10–20 times.

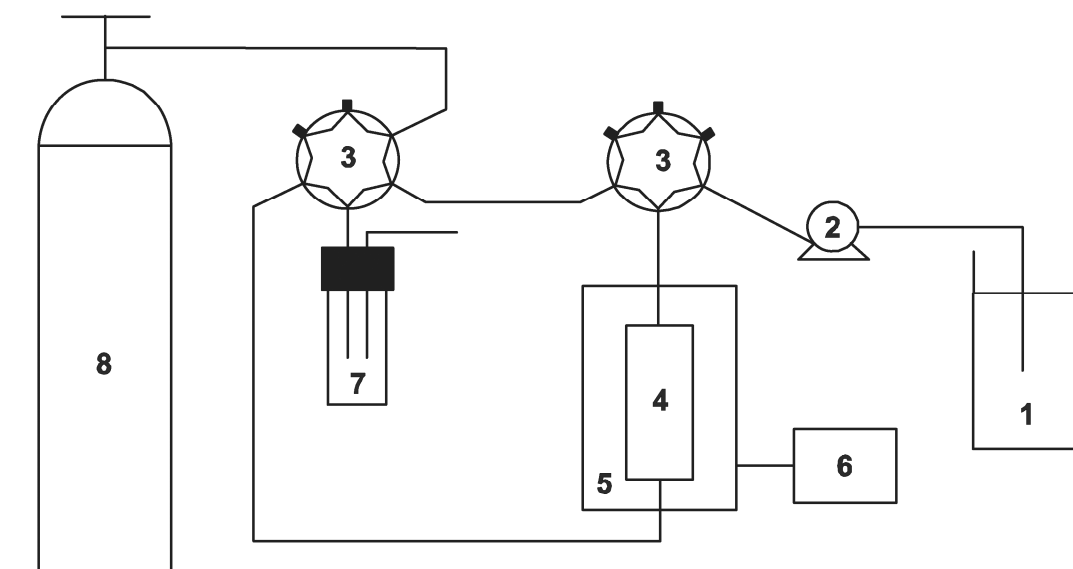


Fig. 1. Scheme of the apparatus. 1, solvent reservoir; 2, high pressure pump; 3, six port valve; 4, extraction cell; 5, heated block; 6, temperature regulator; 7, collecting vessel; 8, gas cylinder.

In this way, the analytes prone to resorption move down through the matrix (similar to frontal chromatography), and are gradually transferred to the collection vial. Finally, the extraction is followed by the flushing steps as described above.

The OnePSE extractor was used for the extraction of hop products by organic solvents at elevated temperature (max. 150°C) and pressure (max. 15 MPa). The use of a solvent mixture containing acetic acid was made possible by changing relevant parts of the extractor for new ones

made from a special material (INCONEL 625). This type of device allows one to use, in accordance with standard extraction method (EPA 3545), a special semidynamic mode, when only a defined part of the extraction volume is discharged and the vessel is immediately replenished.

Sample preparation

With both the Pressurized Solvent Extraction (PSE) and the official extraction method EBC 7.7, different lots

Table I. PSE method. Effect of the inert matrix on the recovery of bitter acids (the hop cones or pellets were ground and mixed with inert material, solvent methanol).

Type of inert matrix	co-humulone (% [m/m])	n+ad- humulone (% [m/m])	α -acids (% [m/m])	α -recoveries (%)	co-lupulone (% [m/m])	n+ad- lupulone (% [m/m])	β -acids (% [m/m])	β -recoveries (%)
No additive	1.27	4.17	2.83	38.51	2.65	2.78	2.83	66.21
Balotina 7	1.12	3.73	2.63	35.76	2.48	2.77	2.85	66.66
Ottawa sand	10.51	30.92	2.90	82.89	27.44	40.97	4.79	87.92
Ottawa sand	10.48	30.80	2.87	81.97	27.72	41.65	4.82	88.46
Sigma-Aldrich sand	10.34	29.93	2.86	81.86	27.72	41.41	4.92	90.25
Sigma-Aldrich sand	10.16	29.76	2.77	79.14	27.31	40.39	4.70	86.17
KORFU-Sidari sand	10.09	29.65	2.79	79.64	26.17	39.47	4.60	84.48
KORFU-Sidari sand	9.57	27.99	2.65	75.79	24.67	37.55	4.39	80.64
Speed matrix	1.20	4.14	2.91	39.55	0.93	1.01	1.05	24.70
No additive	0.65	1.91	2.57	31.18	1.56	2.35	3.91	76.88
Balotina 7	0.60	1.77	1.28	66.87	1.62	2.53	2.24	78.89
Ottawa sand	2.21	8.53	7.52	85.06	1.97	2.37	3.03	91.73
Ottawa sand	2.24	8.60	7.61	86.10	1.98	2.40	3.07	92.98
Sigma-Aldrich sand	2.23	8.51	7.48	84.61	1.98	2.38	3.03	91.80
Sigma-Aldrich sand	2.22	8.49	7.56	85.50	1.93	2.38	3.04	92.02
KORFU-Sidari sand	2.18	8.30	7.14	80.81	1.89	2.35	2.89	87.40
KORFU-Sidari sand	2.04	7.76	6.93	78.42	1.74	2.22	2.80	84.77
Speed matrix	0.12	0.50	0.34	17.77	0.29	0.48	0.42	14.77

Table II. PSE method. Effect of the solvent on the recovery of bitter acids (the hop pellets sample was ground and mixed with Sigma-Aldrich sand inert material).

Solvent	Sample no.	co-humulone (% [m/m])	n+ad- humulone (% [m/m])	α -acids (% [m/m])	α -recoveries (%)	co-lupulone (% [m/m])	n+ad- lupulone (% [m/m])	β -acids (% [m/m])	β -recoveries (%)
Methanol	2443	8.00	23.51	2.40	91.69	22.63	24.39	3.59	91.24
		7.99	23.70	2.43	92.59	22.76	24.61	3.63	92.28
Acetone	2443	8.12	23.78	2.39	91.06	23.44	25.72	3.68	93.58
		7.72	22.66	2.33	88.77	22.32	24.26	3.57	90.76
Ethanol	2443	4.03	13.62	6.87	93.44	5.00	4.97	3.88	90.82
Isopropanol	2443	8.22	24.27	2.45	93.36	24.09	26.49	3.81	96.94
		8.10	23.74	2.43	92.82	23.77	25.85	3.79	96.44
Petrolether	2443	8.78	25.99	2.68	100.91	24.29	24.33	3.74	96.05
		8.83	26.48	2.73	102.94	25.25	25.55	3.93	100.83
		8.97	26.84	2.76	104.25	25.73	25.86	3.98	102.26
	1684	11.34	39.33	3.83	108.89	17.17	20.19	2.82	92.62
		10.65	36.89	3.67	104.16	14.23	16.67	2.38	78.12
		10.81	37.22	3.69	104.89	13.60	15.93	2.27	74.43
	3855	20.52	66.44	6.67	96.59	7.75	6.43	1.09	60.42
		21.15	68.58	6.88	99.58	7.92	7.09	1.15	63.95
		20.50	66.53	6.72	97.23	8.05	7.29	1.18	65.76
Diethylether	2443	9.79	28.22	2.91	110.99	27.00	29.73	4.34	110.44
		10.48	30.40	3.05	116.20	28.57	31.47	4.47	113.79
		9.12	26.79	2.78	104.73	26.38	27.88	4.19	107.72
MeOH/DiEth	2443	9.02	26.50	2.74	103.26	26.80	28.21	4.24	108.90
		8.72	25.25	2.63	99.16	25.48	26.86	4.05	104.03
		8.40	24.28	2.52	95.07	24.77	25.92	3.91	100.40
	1684	10.66	35.03	3.53	100.30	19.08	21.66	3.15	103.19
		9.89	34.13	3.37	95.63	19.37	21.88	3.15	103.41
		9.94	34.24	3.40	96.71	19.70	22.25	3.23	105.95
	3855	19.90	63.87	6.47	93.64	11.63	11.45	1.78	98.99
		20.00	64.22	6.50	99.76	11.68	11.56	1.79	99.68
		21.23	68.18	6.89	99.76	12.23	12.16	1.88	104.41

of hop samples (hop cones, pellets and hop extract) were homogenized and a part of the samples were ground.

PSE method

The samples were accurately weighed in the range of 0.5, 1.5, 2.0 and 3.0 g and mixed with 10 mL inert material (diatomaceous earth, sea or river sands or glass beads). This was important for keeping good solvent permeability and also for water moisture removal. The samples were packed into a 22 or 33 mL vessel made from a highly resistant material (INCONEL 625, Switzerland) and then placed in the OnePSE extractor. The PSE extract was diluted to 50 mL, from which a 10 mL aliquot was put into a 50 mL flask, and the volume was made up to the mark with methanol. From this standardized solution, 10 μ L portions were filtered through a PTFE disc (Whatman, PURADISC 25TF) and injected into the HPLC system.

Official EBC 7.7 method

The hops and hop pellets were ground in a suitable lab mill. Finely ground sample (10 g) was placed into a 250 mL flask and the 20°C attemperated solvents methanol (20 mL), diethylether (100 mL) and 0.1M HCL (40 mL), were added. The mixture was intensively shaken for 40 min and left to stand for 10 min at 20°C for phase separation. The hop extracts were homogenized by stirring. The sample (0.5 g) was placed into a 100 mL flask and 40 mL of 20°C attemperated methanol was added and the extract dissolved for about 30 sec using an ultrasonic bath. The contents were made up to volume with methanol, stoppered and mixed by inversion.

A 5.0 mL aliquot (10 mL for the hop extracts) of the supernatant phase was collected and put into a 50 mL flask and the volume was made up to the mark with methanol, stoppered and carefully mixed. The solution was filtered using a 0.45 μ m microfilter and injected into the HPLC system.

HPLC-UV VIS

The filtered extraction solutions were analysed by the HPLC chromatographic system TSP1000 (Thermo Separations Products), equipped with UV diode array detector. HPLC analysis was performed using a stainless steel column packed with C18 Hop, 5 μ m, 250 \times 4 mm (Macherey Nagel), with NucleoSil 100-5 precolumn C18 Hop connected between the injection port and the main column. The separation of α and β -acids was carried out in isocratic mode; the column temperature was kept at 35°C. The mobile phase was prepared by mixing 850 mL methanol + 190 mL water + 5 mL ortho-phosphoric acid; the flow rate was set at 0.9 mL/min. The eluted bitter acids were detected at 314 nm and the time needed for one analysis was about 23 min. The particularity and selectivity was guaranteed by using an HPLC column made especially for the analysis of bitter acids and no interference was observed. The calibration of the method was carried out using the international calibration standard ICE 2. The official EBC method which uses solid-liquid extraction of hop samples with diethylether-methanol-hydrochloric acid was used as a comparative method for calculating the recovery.

RESULTS AND DISCUSSION

There are relatively many experimental parameters influencing the extraction efficiency with different levels of significance. The most important parameters include temperature, type of extraction solvent, sample preparation procedure and the number of cycles. Optimization of these parameters was carried out in the first part of this work. As was found in the first set of preliminary experiments, the critical parameter was sample preparation (grinding) together with mixing with an appropriate inert material. The influence of grinding and mixing the samples with different types of inert additives is illustrated in Table I. The milling of samples (pellets) allowed us to reach a recovery 38% for α -acids and 66% for β -acids, and the subsequent mixing with sand further increased the recoveries up to values in the 90% area.

One of the most important parameters influencing the recoveries in PSE is a proper choice of inert matrix. With respect to good results and availability of the Sigma-Aldrich sand, all subsequent experiments were carried out with this inert additive, while the very poor results with Speed matrix (Table I) entirely excluded this popular additive from further work.

Another important factor is the solvent or solvent mixture. The influence of the solvent used for extraction is depicted in Table II. The best results were achieved by using pure diethylether. However, problems with pumping and with high flammability of this compound led us to use a 1:1 MeOH/DiEth (v/v) mixture as the optimum extraction solvent.

The optimum extraction conditions arising from the optimization experiments were applied to a set of samples with a varying quantity of bitter acids. Table III shows the optimized extraction conditions.

Tables IV and V show that the method is effective for both low-alpha and high-alpha hop cones as well for hop extracts (Table VI). Recoveries between 96.8 and 102.7% were achieved for all samples.

Compared with the standard method EBC 7.7, the PSE method is less laborious and time consuming and brings large savings of solvents. The extraction time was shortened from 50 min (EBC 7.7) to 15 min (PSE) and instead of a 160 mL of extraction mixture (EBC 7.7) the new proposed extraction procedure only uses a 40 mL 1:1 MeOH/DiEth mixture (Table VII). Additionally, applying the automatic FastPSE extraction, the sample throw is increased by six times (Table VII).

Table III. PSE method. Optimized extraction conditions.

Number of cycles	3
Cycle size	5 min
Temperature	80°C
Mode	A
Pressure	15 MPa
Quantity	1.5 g of ground hop cones or pellets or 0.3 g of hop extract
Solvent	methanol–diethylether (1:1)
Inert matrix	sea sand (50 to 70 μ m)
Vessel volume	22 mL (ground cones and pellets or extract + 10 mL inert matrix)
Solvent rinsing	20 sec
Nitrogen blow down	2 min

Table IV. Comparison of PSE and EBC 7.7 methods – low-alpha hop cones.

Sample no.	α -acids PSE (% [m/m])	α -acids EBC (% [m/m])	α -acids recovery (%)	β -acids PSE (% [m/m])	β -acids EBC (% [m/m])	β -acids recovery (%)
1	1.91	1.92	99.5	3.89	3.93	99.0
2	1.98	1.98	100.0	4.43	4.42	100.2
3	2.17	2.20	98.6	3.90	3.93	99.2
4	2.23	2.19	101.8	4.68	4.61	101.5
5	2.07	2.01	103.0	4.13	4.11	100.5
6	1.51	1.56	96.8	3.49	3.55	98.3
7	1.89	1.85	102.2	3.95	3.96	99.7
8	1.82	1.82	100.0	4.17	4.16	100.2
9	1.22	1.25	97.6	3.60	3.62	99.4

Table V. Comparison of PSE and EBC 7.7 methods – high-alpha hop cones.

Sample no.	α -acids PSE (% [m/m])	α -acids EBC (% [m/m])	α -acids recovery (%)	β -acids PSE (% [m/m])	β -acids EBC (% [m/m])	β -acids recovery (%)
1	9.12	9.07	100.6	6.47	6.34	102.1
2	8.88	8.90	99.8	6.30	6.36	99.1
3	9.23	9.28	99.5	5.72	5.65	101.2
4	5.24	5.34	98.1	5.48	5.62	97.5
5	7.85	7.84	100.1	4.90	4.84	101.2
6	7.25	7.34	98.8	4.32	4.42	97.7
7	6.96	7.09	98.2	3.54	3.53	100.3
8	7.46	7.44	100.3	3.81	3.71	102.7
9	10.23	10.27	99.6	6.32	6.24	101.2

Table VI. Comparison of PSE and EBC 7.7 methods – hop extracts.

Sample no.	α -acids PSE (% [m/m])	α -acids EBC (% [m/m])	α -acids recovery (%)	β -acids PSE (% [m/m])	β -acids EBC (% [m/m])	β -acids recovery (%)
1	59.28	59.31	99.9	22.95	22.94	100.0
2	59.13	59.03	100.2	22.78	22.72	100.3
3	28.11	28.05	100.2	10.75	10.75	100.0
4	28.68	28.78	99.7	11.09	11.05	100.4
5	59.46	59.53	99.9	23.00	22.88	100.5
6	59.15	59.12	100.1	21.89	22.10	99.0

Table VII. Comparison of extraction methods

	Method		
	EBC 7.7	OnePSE	FastPSE
Extraction type	Automatic	Semiautomatic	Automatic
Extraction time (min)	50	15	15
Extraction solvent mixture (mL)	160	40	40
Equipment	Shaker	OnePSE extractor	FastPSE extractor
Equipment price	1300 €	18 000 €	39 000 €
Samples per run	4	1	6
Samples per hour	4	4	24

Table VIII. Shortened PSE method – hop pellets.

Solvent mix	Sample no.	α -acids PSE (% [m/m])	α -acids EBC 7.7 (% [m/m])	α -acids recoveries (%)	β -acids PSE (% [m/m])	β -acids EBC 7.7 (% [m/m])	β -acids recoveries (%)
MeOH/DiEth							
1:1	1633	3.89	3.75	103.70	3.45	3.32	103.39
1:1	974	3.23	3.21	101.49	7.03	6.96	102.53
1:1	4083	6.58	6.98	93.81	4.00	4.04	96.86
1:1	1633	3.89	3.75	103.70	3.45	3.32	103.39
1:3	1633	3.75	3.75	100.00	3.38	3.32	101.86
1:5	1633	3.90	3.75	104.10	3.65	3.32	108.90

In order to increase the sample throughput, the extraction time was reduced to 3 consecutive 1- min cycles (3 × 1 min) and various extraction mixtures were chosen. As shown in Table VIII, the static extraction time of 1 min using a 1:1 MeOH/DiEth (v/v) mixture was sufficient for

most samples except for sample No. 4083, which contained a much higher concentration of bitter acids than the other samples.

To keep a high efficiency of extraction, even for the highly concentrated samples, a static time of 3 × 5 min is

Table IX. Results of statistical comparison of PSE and EBC 7.7 methods.

Samples	α -acids						β -acids					
	Paired test		Sign test		Ranking test		Paired test		Sign test		Ranking test	
	P	P _{crit}	P	P _{crit}	P	P _{crit}	P	P _{crit}	P	P _{crit}	P	P _{crit}
Low-alpha hops	1	0.05	0.723670	0.05	0.999994	0.05	0.673286	0.05	0.999994	0.05	0.510976	0.05
High-alpha hops	0.088149	0.05	0.504983	0.05	0.122875	0.05	0.613908	0.05	0.504983	0.05	0.593628	0.05
Hop extract	0.921825	0.05	0.999994	0.05	0.999994	0.05	0.973424	0.05	0.617072	0.05	0.787402	0.05

recommended for quantitative extraction of bitter acids from pellets, hop cones and hop extracts at all concentration levels.

The last phase of this work was to compare the official EBC method 7.7 with PSE and to carry out a statistical evaluation. As shown in Table IX, in all tests the p-value was greater than p_{crit} so that we cannot reject the null hypothesis at the 95% confidence level. Therefore, the newly proposed PSE method can be considered as fully comparable with the classical EBC 7.7 extraction method.

ACKNOWLEDGEMENTS

The authors thank the members of the Czech Beer and Malt Association, the Ministry of Education, Youth and Sports of the Czech Republic (Research Centre 1M0570 and MSM 6019369701), the Czech Science Foundation (Project GA203/08/1536) and the Academy of Sciences of the Czech Republic (Institutional Research Plan No. AV0Z40310501) for financial support.

REFERENCES

1. Analytica EBC, European Brewery Convention, Method 7.4, 5th. Edition, Verlag Hans Carl Getränke-Fachverlag: Nürnberg, 1998.
2. Analytica EBC, European Brewery Convention, Method 7.7, 5th. Edition, Verlag Hans Carl Getränke-Fachverlag: Nürnberg, 1998.
3. Analytica EBC, European Brewery Convention, Method 7.8, 5th. Edition, Verlag Hans Carl Getränke-Fachverlag: Nürnberg, 1998.
4. Analytica EBC, European Brewery Convention, Method 7.9, 5th. Edition, Verlag Hans Carl Getränke-Fachverlag: Nürnberg, 1998.
5. Anonymous. EPA method 3545A, U.S. Environmental Protection Agency, Revision 1, Jan. 1998.
6. Brautechnische Analysenmethoden, Band. I., MEBAK, Freising, 1997.

7. Chen, X. J., Guo, B. L., Li, S. P., Zhang, Q. W., Tu, P. F. and Wang, Y. T., Simultaneous determination of 15 flavonoids in epimedium using pressurized liquid extraction and high-performance liquid chromatography. *J. Chromatogr. A*, 2007, **1163**(1-2), 96-104.
8. Daoud, I.S. and Kusinski, S., Liquid CO₂ and ethanol extraction of hops. 1. Effect of hop deterioration on extraction efficiency and extract quality. *J. Inst. Brew.*, 1992, **98**(1), 37-41.
9. Daoud, I.S. and Kusinski S., Liquid CO₂ and ethanol extraction of hops. 2. Effect of hop deterioration on the time course of extraction. *J. Inst. Brew.*, 1993, **99**(1), 39-41.
10. Daoud, I.S. and Kusinski S., Liquid CO₂ and ethanol extraction of hops. 3. Effect of hop deterioration on utilization and beer quality. *J. Inst. Brew.*, 1993, **99**(2), 147-152.
11. del Valle, J. M., Rivera, O., Teuber, O. and Palma, M. T., Supercritical CO₂ extraction of Chilean hop (*Humulus lupulus*) ecotypes. *J. Sci. Food Agric.*, 2003, **83**(13), 1349-1356.
12. IOB Methods of Analysis, Institute of Brewing, 6.4, Vol. 1 – Analytical, IOB: London, 1997.
13. IOB Methods of Analysis, Institute of Brewing, 6.5, Vol. 1 – Analytical, IOB: London, 1997.
14. Langezaal, C. R., Chandra, A., Katsiotis, T. S., Scheffer, J. J. C. and Dehaan, A. B., Analysis of supercritical carbon-dioxide extracts from cones and leaves of a *Humulus-lupulus* L cultivar. *J. Sci. Food Agric.*, 1990, **53**(4), 455-463.
15. Smelcerovic, A., Spittler, M. and Zuehlke, S., Comparison of methods for the exhaustive extraction of hypericins, flavonoids, and hyperforin from *Hypericum perforatum* L. *J. Sci. Food Agric.*, 2006, **54**(7), 2750-2753.
16. Waksmundzka-Hajnos, M., Wianowska, D., Oniszczuk, A. and Dawidowicz, A. L., Effect of sample-preparation methods on the quantification of selected flavonoids in plant materials by high performance liquid chromatography. *Acta Chromatogr.*, 2008, **20**(3), 475-488.
17. Zhang, Y., Li, S. F. and Wu, X. W., Pressurized liquid extraction of flavonoids from *Houttuynia cordata* Thunb. *Separation and Purification Technol.*, 2008, **58**(3), 305-310.

(Manuscript accepted for publication September 2009)