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Novel Application of Square-Wave Adsorptive-Stripping Voltammetry for the Determination of Xanthohumol in Spent Hops

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ABSTRACT: This paper reports the development of a novel electrochemical assay for xanthohumol (XN) by square-wave adsorptive-stripping voltammetry (SWAdSV) with a hanging mercury drop electrode. The method showed good repeatability (CV < 2%) and linearity (between 10 and 250 $\mu g\ L^{-1}$), as well as suitable limits of detection (2.6 $\mu g\ L^{-1}$) and quantification (8.8 $\mu g\ L^{-1}$). The method was applied for the quantification of this compound in spent hops, and the results obtained were compared with the HPLC-UV method. XN contents determined by the SWAdSV method were 16 ± 1 and $100 \pm 4\ \mu g\ L^{-1}$ for aqueous and methanolic extracts, respectively. The developed new methodology considerably reduces the analysis time, approximately from 25 min (HPLC-UV method) to 7 min, enabling a high sample throughput. In addition, the detection and quantification limits were approximately 5-fold lower than those obtained with the chromatographic method.

KEYWORDS: square-wave adsorptive-stripping voltammetry, xanthohumol, spent hops, electrochemistry

INTRODUCTION

The hop plant (*Humulus lupulus* L.) is a dioecious perennial plant of the Cannabacea family, cultivated in most temperate zones of the world for its female inflorescences. The plant has been used in the brewing industry for a long time to add bitterness and aroma to beer.[1,2] Hops are very rich sources of prenylflavonoids, which are secreted along with bitter acids and essential oils by the lupulin glands of the inflorescences.[3] Besides hop's usefulness in the beer industry, hop components exhibit many pharmacological activities, the antioxidant action being one of the most promising. The most important studied phenolic compound of this raw material has been the prenylated chalcone xanthohumol (XN). This compound is the main prenylflavonoid of hops (0.2−1.1% in dried hops) and has received the most attention in recent years. XN has been found to have a range of interesting biological properties in vitro that may have therapeutic utility including hormonal (for relief of "hot flashes" and treatment of osteoporosis),[4] antioxidant (for treating atherosclerosis),[5] and inhibition of HIV-1.[6] This compound has also a multimechanism classification as a potential "broad-spectrum" anticancer and cancer prevention agent (applicable to both breast and prostate cancers).[7,8] In light of the potential use of XN in medicine, as well as the importance of this hop component in the brewing sector, sensitive and expeditious methods for the determination of XN are needed.

The analysis of XN in hops is generally performed by high-performance liquid chromatography (HPLC) coupled with ultraviolet/diode array or mass spectrometry detection.[2,9] The widespread usage of HPLC methods is justified by the high sensitivity and the possibility of the simultaneous analysis of the XN and related prenylflavonoids, which increases its usage even further. Kac and co-workers[10] developed a HPLC method with coulometric detection for the quantification of XN, α-acids, and β-acids in hops. The method developed by these authors was accurate and very sensitive for the detection of these compounds, with detection limits at least 8.8 times lower when compared to ones obtained using HPLC with UV detection, although the relatively long analysis times and the high acquisition and maintenance costs make this technique inappropriate for routine analysis. Several other methods have also been described. Kac and co-workers[11] developed a method based on nonaqueous capillary electrophoresis for the analysis of XN, but the proposed method is neither precise nor accurate. Vanhoenacker et al.[12] used microemulsion electrokinetic chromatography for determination of XN in the presence of other hop components. However, the pH of the electrolyte was of outmost importance, and slight pH changes affected dramatically the separation. Another method used was capillary electrochromatographic analysis that proved to be sensitive to XN and reduces the effects of slight pH variations, but the authors reported poor repeatability in their system.[13]

Therefore, it is extremely important to develop new analytical methodologies that provide good repeatability, high sample throughput, high sensitivity, and low maintenance costs.

Adsorptive stripping voltammetry is a highly sensitive and selective technique for the analysis of organic compounds, which can be accumulated at the hanging mercury drop electrode (HMDE) surface and afterward stripped off by applying a potential scan.[14] This allows their selective preconcentration at the electrode; also, the introduction of high scan rate voltammetric techniques, such as square-wave voltammetry (SWV), allows the sensitivity of adsorptive stripping voltammetry to be increased even further.[15,16] The previous concepts were used in the development and validation of a rapid and reliable analytical methodology for the quantification of XN in spent hops

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(byproduct resulting from hop's supercritical CO_2 extraction) by square-wave adsorptive-stripping voltammetry (SWAdSV). To our knowledge, this is the first application of voltammetry for the quantification of XN. Furthermore, our investigation was focused on the quantitative determination of this compound in spent hops.

MATERIALS AND METHODS

Reagents. XN (90%) standard was kindly supplied by Hopsteiner (Mainburg, Germany). A stock standard solution (500 mg L^{-1}) of this compound was prepared by rigorous dissolution of 0.0125 g of the analyte in methanol (25 mL). The standard solution was stored at 4 °C and used for further dilutions. All other reagents used were of analytical grade and were purchased from Merck and Sigma-Aldrich. High-purity water from a Millipore Simplicity 185 water purification system (Millipore Iberian S.A., Madrid, Spain) was used for all chemical analyses and glassware washing. The solvents employed for HPLC analysis were filtered through a cellulose filter of 0.45 μm pore size (Whatman, Clifton, NJ) and degasified for 10 min in an ultrasound bath.

Voltammetric Procedure. Voltammetric measurements were performed using a Metrohm 663VA voltammetric stand (Herisau, Switzerland) equipped with a HMDE, drop size of 0.024 mm^3, a glassy carbon auxiliary electrode, and a reference Ag/AgCl, 3 mol L^{-1} electrode. The system was connected to an Autolab PGSTAT12 voltammetric system (Metrohm-Eco Chemie), controlled by a PC with the GPES 4.9 software (Metrohm-Eco Chemie). The cyclic voltammogram for a 250 μg L^{-1} XN standard was obtained in the potential range from −1.5 to 0 V at a scan rate of 100 mV s^{-1}. SWAdSV was performed using 20.0 mL of the supporting electrolyte (0.1 mol L^{-1} ammonium buffer solution, pH 8.8), deoxygenated with water-saturated pure nitrogen for 300 s. An accumulation potential (E_{acc}) of −0.4 V was applied for 60 s with stirring, followed by a potential scan from −1.0 to −1.5 V (frequency, 100 Hz; pulse step, 4 mV; pulse amplitude, 20 mV). After recording of the background voltammogram, an aliquot of the standard or sample solution was added and the procedure was repeated using a purge time of 10 s. The analysis time of each sample was 7 min, and all measurements were made at room temperature.

The developed procedure for the determination of XN in spent hops was validated using the recommendations of the International Union of Pure and Applied Chemistry (IUPAC).[17] The validation parameters determined were linearity, limit of detection (LOD), limit of quantification (LOQ), precision, recovery, and selectivity.

Chromatographic Analysis. The chromatographic analyses of XN were carried out following the procedure described by Magalhães et al.[2] Separations were achieved on a Varian (Varian Inc., Palo Alto, CA) Nucleosil C_{18} column (250 mm × 4.6 mm, 5 μm), and the flow rate was 0.8 mL min^{-1}. The detection wavelength of XN was 368 nm, and 20 μL of sample was injected onto the column that was kept at room temperature. The analysis of each sample was conducted with a running time of 25 min, and the peak of XN was identified by comparing its retention time ($t_R \approx 11$ min) and UV−vis spectra with those of standard solutions. Peak purity was checked to exclude any contribution from interfering peaks.

Spent Hops Extraction Procedure. The adequacy of the developed method was evaluated by quantifying XN in spent hops extracts. For the analysis, the byproduct recovered from supercritical CO_2 extraction of hops, commonly used by brewers, was used in this work. Spent hop product was finely ground in a laboratory EBC mill (Casela, London, U.K.), and XN's extraction was tested for two solvents (methanol and water) by applying ultrasonication. Ground sample (0.1 g) was extracted with 10.0 mL of each solvent on an ultrasonic bath (Cole-Parmer, model 8891) at 40 kHz for 20 min. After centrifugation (3500*g*, 5 min), the remaining byproduct was re-extracted for 20 min using 10.0 mL of fresh extraction solvent. The extracts obtained were combined for use afterward. All of the extracts were filtered through a 0.2 μm cellulose membrane filter (Schleicher & Schuell, Microscience) and homogenized.[2]

For voltammetric analysis 20 μL of the methanolic extract (4-fold diluted) was added to 20.0 mL of the electrolyte solution contained in the voltammetric cell. Four standard additions between 0 and 50 μg L^{-1} were made by adding increasing quantities of XN standard to the sample solution contained in the voltammetric cell. This procedure was repeated in triplicate for each extract. Aqueous extract was analyzed without further dilution.

As for voltammetric analysis, the methanolic extract was 10-fold diluted before analysis by HPLC-UV and the aqueous extract was analyzed without further dilution.

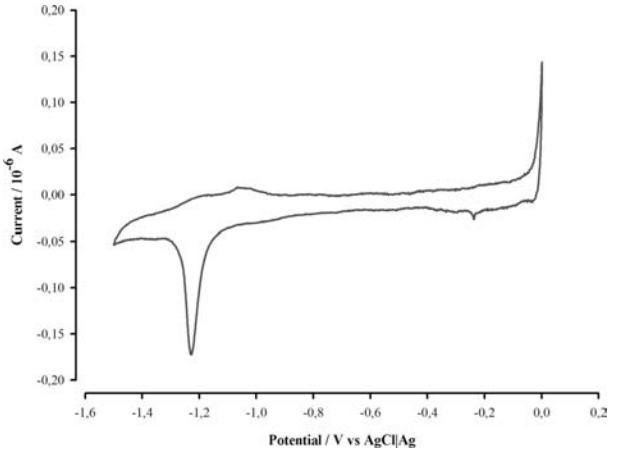

Figure 1. Cyclic voltammogram of an XN standard solution (250 μg L^{-1}) in pH 8.8 ammonium buffer scanning in a positive direction from −1.5 to 0 V (scan rate, 100 mV s^{-1}; step potential, 1.98 mV).

RESULTS AND DISCUSSION

The quantification of XN using a very sensitive SWAdSV method presents an alternative to the classical HPLC-UV method,[2] especially when samples with low content of the considered compound are under investigation. The literature data regarding the analytical methods for this compound are fairly poor, and except for the expensive LC-MS method,[9] no other methods with LOD below 10 μg L^{-1} are reported. As the studied compound tends to have some redox activity, the SWAdSV technique was chosen and compared with the well-known and commonly used HPLC method.

Cyclic Voltammetry. The choice of the proper potential for the analyte detection requires a compromise between sensitivity and selectivity. In view of these considerations, cyclic voltammograms of the studied compound were acquired by scanning in a positive direction from −1.5 to 0 V and back to −1.5 V. This enabled us to select the optimal potential to get a maximal response for XN's reduction. A representative voltammogram is shown in Figure 1. As can be seen, the cyclic voltammogram of a XN standard solution (250 μg L^{-1}), in pH 8.8 ammonium buffer recorded with a scan rate of 100 mV s^{-1}, exhibited one well-defined cathodic peak at −1.2 V and no reversed peak was observed. The reduction wave appears to correspond to an irreversible process, as no current is observed in the reverse scan. The mechanism of reduction of XN on a HMDE using cyclic voltammetry is still under investigation in order to evaluate the electrochemical behavior of this compound.

Optimization of the SWAdSV Procedure. The electrochemical behavior of XN was studied under batch conditions by SWAdSV. For optimization of electrolyte pH, several buffers, such as phosphate, acetate, and ammonium, were used to cover a wide pH range (1.1−12.1). Figure 2 represents the dependence of peak current intensity (i_p) on pH for two XN concentrations. The stripping wave (E_{acc} −0.50 V; t_{acc} 30 s) obtained, using a frequency of 100 Hz, increased with pH in the range 1.1−8.8. Because the highest i_p was provided by a pH 8.8 ammonium buffer (0.1 mol L^{-1}), the other parameters governing this method were studied and optimized to find the best analytical signal for the analysis of XN at this pH. The dependence of the stripping peak current on E_{acc} between −0.1 and −1.1 V was studied for a standard solution of XN (50 μg L^{-1}) using a 30 s accumulation time (t_{acc}) (Figure 3I). A maximum i_p was obtained for E_{acc} between −0.4 and −0.6 V. An E_{acc} of −0.4 V was adopted and used in the present study. The effect of t_{acc} on i_p for a standard solution of XN of 50 μg L^{-1} was then studied (Figure 3II), showing a linear behavior up to approximately 1 min (Figure 3II), after which the increase of i_p is lower, possibly due to saturation coverage of the drop and/or competitive adsorption.

Several experimental conditions related to the square-wave potential scan, such as frequency, pulse step (ΔE_s), and pulse amplitude (ΔE_p) were then studied and optimized to obtain maximum signal-to-noise ratio and repeatability. These conditions are interrelated and have a combined effect on the analytical signal. The influence of frequency was studied in the range of 25−1000 Hz, and the peak current increased linearly between 25 and 800 Hz. However, the poor definition of the XN reduction peak for frequency >100 Hz and the fact that the process is irreversible make the use of high frequency unnecessary. Therefore, a frequency of 100 Hz was adopted in subsequent experiments. Variation of ΔE_s (Figure 4I) from 1 to 8 mV resulted in a marked increase of i_p up to 4 mV, which increased less significantly for higher values. A pulse step of 4 mV was chosen for analysis. Although i_p increased with ΔE_p (Figure 4II) up to 50 mV, a 20 mV pulse amplitude was adopted because with this value better peak definition was obtained.

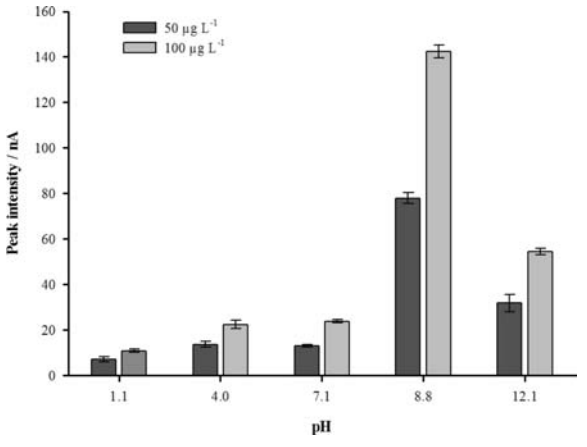

Figure 2. Dependence of peak current intensity (i_p) on pH for two XN concentration levels.

Validation of the SWAdSV Procedure. When the optimum experimental conditions were found, validation of the method was conducted.

To test the influence of the matrix, the calibration curve and standard additions method were performed in the methanolic extract. By the analysis of slopes obtained for both methods (calibration curve, 1.23 ± 0.01 nA L μg^{-1}; standard addition, 1.45 ± 0.03 nA L μg^{-1}) it was concluded that the matrix has an influence on the determination of XN. Therefore, it was decided to evaluate the method features using the standard additions of XN to the methanolic extracts (Figure 5). The linearity was verified by spiking the methanolic extract with the XN standard in the range of 10−250 μg L^{-1}. A calibration curve was constructed by plotting i_p as a function of the standard increment, and a good linearity was achieved (correlation coefficient, r = 0.9996) in the tested range. Furthermore, LOD (2.6 μg L^{-1}) and LOQ (8.8 μg L^{-1}) were evaluated on the basis of the signal obtained in the analysis of the sample with standard additions of XN (n = 5), following the recommendations of IUPAC. Moreover, the method's precision was determined by measuring repeatability (intraday variability) and intermediate precision (interday variability) of the peak intensity for the methanolic extract. The precision was evaluated as the coefficient of variation (CV) for five repeated analyses of the sample in the same day (intraday precision). The interday experiments were performed on three validation days and repeated five times within each day. The results showed that the intraday CV (1.6%) and the interday CV (2.5%) were <3%, showing that

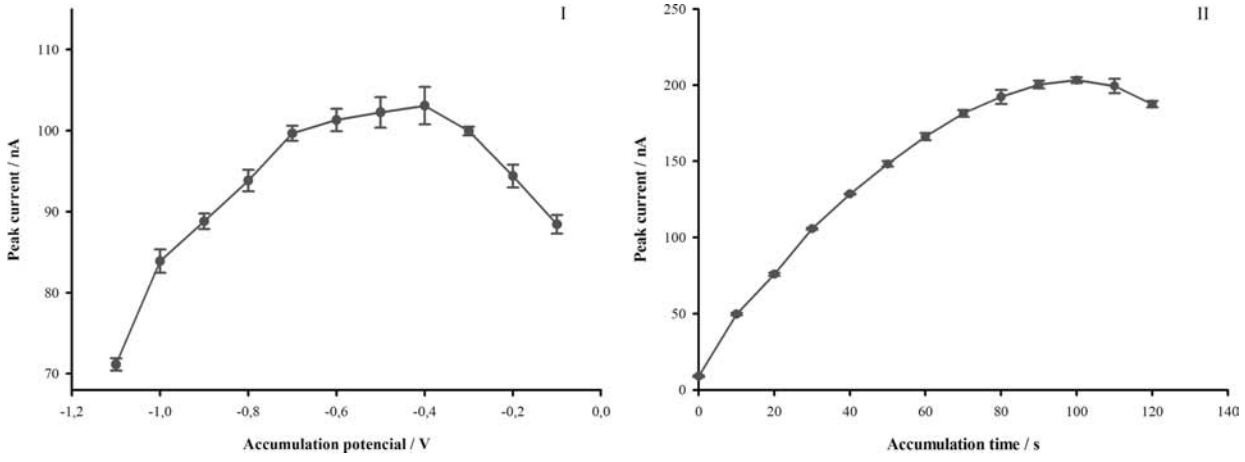

Figure 3. Effect of accumulation conditions on peak current of a standard XN solution (50 μg L^{-1}) in a pH 8.8 ammonium buffer: (I) effect of E_{acc} on peak current, t_{acc} = 30 s; (II) effect of t_{acc} on peak current, E_{acc} = −0.4 V.

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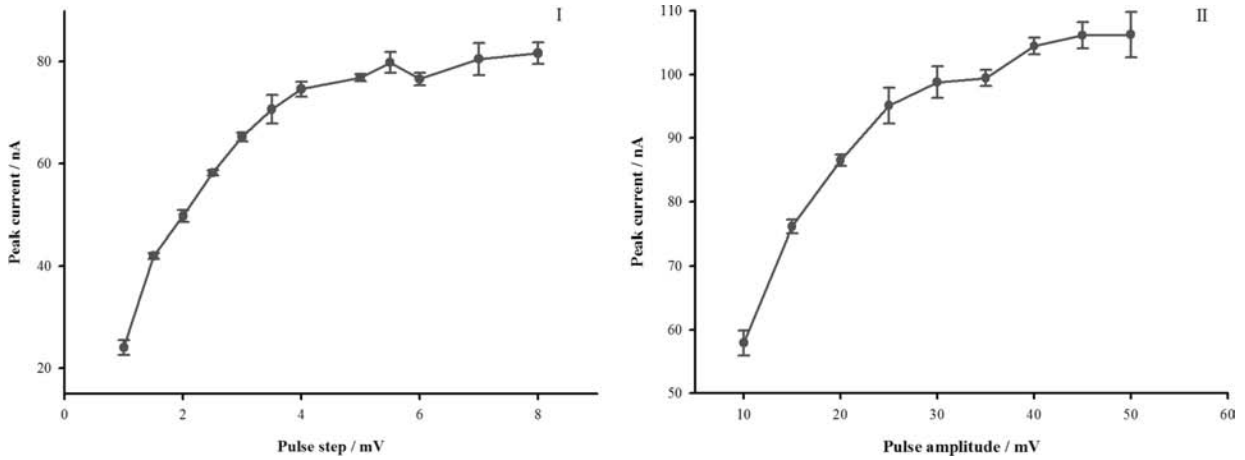

Figure 4. Effect of pulse step (I) and pulse amplitude (II) on peak current of a standard XN solution (50 μg L^{-1}) in a pH 8.8 ammonium buffer.

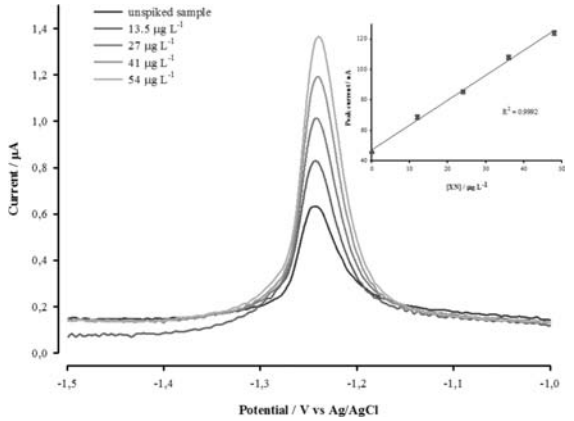

Figure 5. Square-wave adsorptive-stripping voltammograms obtained in the determination of XN in methanolic extract using the standard additions method (standard additions of XN ranging from 0 to 50 μg L^{-1}). (Inset) Plot of the calibration curve obtained.

Table 1. Recovery Studies of the Proposed Method

XN concn[a] (μg L^{-1})	concn added (μg L^{-1})	recovery (%) ($\chi \pm$ SD[b])	CV (%)
25.4 ± 0.9	12	98.2 ± 1.2	1.2
	24	97.5 ± 1.8	1.8
	36	96.3 ± 1.5	1.6
	48	97.7 ± 1.1	1.1

[a] In the 4-fold diluted extract. [b] Standard deviation for the average, n = 3.

the method is precise and also confirming the stability of the spent hop extracts during the evaluation period.

Recovery experiments were performed to study the accuracy of the method (Table 1). Thus, known amounts of XN were added to the methanolic extract, and the resulting spiked samples were subjected to the entire analytical sequence. The sample was spiked at four different concentrations, and recoveries were calculated on the basis of the difference between the total amount determined in the spiked samples and the amount observed in the nonspiked sample. All analyses were carried out in triplicate. The average recoveries obtained were all >96% (Table 1),

Table 2. Comparison of the XN Concentrations Obtained by the Voltammetric and HPLC Methods

	XN concn[a] (μg L^{-1})	
solvent	HPLC method	voltammetric method
water	14.6 ± 0.9	16 ± 1
methanol	98 ± 3	100 ± 4

[a] Values are the mean ± standard deviation, n = 4.

testifying to the accuracy and selectivity of the proposed methodology for the analysis of XN in spent hops extracts.

Application of the SWAdSV Procedure. The optimized procedure was then applied to spent hops extracts. Due to the previously mentioned matrix effect, standard additions were used to quantify the content of XN in the different spent hops extracts (Figure 5). As can be seen in Table 2, the solvent can affect the content of XN in spent hops extracts. Analysis of the results shows that the content of XN was lower in samples extracted with water. XN contents determined by the SWAdSV method were 16 ± 1 and 100 ± 4 μg L^{-1} for the aqueous and methanolic extracts, respectively. The results here obtained are in accordance with those previously reported.[18] Magalhães et al.[2] investigated the influence of the solvents with distinct polarities, such as acetonitrile, methanol, ethyl acetate, hexane, and water, on the extraction of XN and concluded that methanol was the most efficient solvent.

The validation of the SWAdSV method was carried out using the chromatographic method previously described.[2] At least three replicates were done in the samples (methanolic and water extracts) analyzed by both methods. According to the results displayed in Table 2, a good agreement between the proposed new methodology and the chromatographic one (XN contents of 14.6 ± 0.9 and 98 ± 3 μg L^{-1} for aqueous and methanolic extract, respectively) was achieved, confirming the accuracy of the voltammetric method here presented. By applying a statistical test (Student t test), it was confirmed, with a confidence level of 99%, that there are no significant differences between the results obtained by both methodologies. Furthermore, the voltammetric method considerably reduces the time of analysis, approximately from 25 min (HPLC-UV method) to 7 min, enabling a high sample throughput. In addition, the detection and quantification

limits were 5-fold lower than those obtained by the chromatographic method (LOD = 15 μg L^{-1} and LOQ = 49 μg L^{-1}).

In conclusion, this work was aimed at developing a rapid, reproducible, and accurate analysis method for the quantification of XN in spent hops extracts. To our knowledge, this is the first application of voltammetry for the quantification of XN. The study was conducted to compare the classically used HPLC method with our new proposed SWAdSV methodology for quantification of XN in spent hops. The method is characterized by good precision, accuracy, and sensitivity. The detection and quantification limits were about 5-fold lower than those of the chromatographic method. The validated method was successfully applied to quantify XN in the extracts of spent hops and provides a good alternative method to the chromatographic one. Moreover, the new developed SWAdSV method has the advantages of significantly reducing the analysis time and overall costs as well as providing a high sample throughput. Furthermore, the developed new methodology can be adapted in the future in a flow injection analysis (FIA) system as well as for the quantification of XN in the different forms of hops (pellets and extracts) and beer.

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