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Short communication

Elimination of matrix effects for static headspace analysis of ethanol

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Abstract

The intention of this work was to develop a simple and fast procedure for a determination of small amounts of ethanol in aqueous protein containing solutions based on combined headspace gas chromatography. In order to provide for short analysis time static headspace methodology was considered for this purpose. In this context the influence of the matrix composition onto the analytical results has been established and internal standardization as well as a full evaporation technique have been evaluated as promising alternatives for a compensation of matrix effects. With respect to speed of analysis, simplicity of sample handling as well as the quality of the analytical performance parameters, precision and accuracy, the full evaporation technique proved to be superior. Thus, the static equilibration of a 20 μl sample aliquot in a conventional headspace sample vial for 5 min at 100°C is sufficient to obtain equilibrium conditions for gas chromatographic analysis. The accuracy of this method was verified by robust regression analysis and exhibited excellent robustness within the required limits of sample composition ranging from 0 to 20% (w/w) protein content and up to 5 g/l salt content.

Keywords: Headspace analysis; Matrix effects; Regression analysis; Ethanol; Alcohols; Albumin; Proteins

1. Introduction

A method has been developed for the determination of traces of ethanol in aqueous human albumin solutions for the application in pharmaceutical quality control. Ethanol is used as precipitant in the ''Cohn process'' for the stepwise fractionation of human plasma proteins. The preparation of the final product solutions requires an as complete as possible removal of the added alcohol, which is accomplished usually by ultra- or diafiltration [1]. In order to verify the conformity of the procedure and for medicinal reasons the residual trace content of the alcohol in the resulting differently concentrated protein solutions needs to be established precisely. Thus, the objective was to develop a fast and accurate method for routine analyses in pharmaceutical industry.

The most common methods for a trace determination of ethanol in protein containing solutions are provided by enzymatic and headspace gas chromatographic analysis [2–4]. For the present study a static headspace technique has been selected for this purpose. Since the partition coefficients of volatile analytes between the condensed and the gas phase are influenced by the chemical and physical prop-

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erties of the sample, special emphasis has to be given to a proper consideration of the effect of the matrix composition onto the analytical signal. Quantification is merely possible after careful selection of the instrumental parameters and of a suitable calibration method [5,6].

A well established method to compensate for matrix effects in headspace analysis is the use of internal standards. This principle is widely applied for the analysis of volatile substances in biological samples and is accepted in forensic medicine for the measurement of the ethanol content in blood [4,5]. Due to the rather complex nature of the headspace equilibria, internal standard calibration does not generally eliminate the the matrix effect and systematic errors may occur, however [7].

In 1993 Markelov and Guzowski, Jr. established the full evaporation technique to overcome the matrix effects which are not to be avoided with the application of direct headspace injection techniques [8]. The main benefit of the full evaporation technique relates to the so called ''criterion of full evaporation''. It is described by the nearly complete transfer of analytes, not the sample itself, from the condensed matrix into the vapor phase. This criterion can be met by using high equilibration temperature and an adequate small sample size. For method development it is important to consider that the evaporation temperature does not reach a level which results in sample decomposition or a formation of artefacts. The fulfillment of these basic requirements may generally be verified with just a few experiments. The associated elimination of the effect of sample matrix onto the response signal results in the potential for an universal standardization procedure. Some applications of this rather new technique have already been explored for the analysis of volatile organic compounds in pharmaceutical products and biological samples [9,10].

In this work the actual influence of the matrix composition onto the chromatographic response was investigated by regression analysis and the techniques of internal standard calibration and of full evaporation were inspected with respect to their capabilities to eliminate the effect of the sample matrix on quantitation. The optimized procedure has been validated following the the appropriate guidelines [11–13]. Furthermore, the applicability of this method has been tested for a determination of ethanol in so called non-alcoholic beer.

2. Experimental

2.1. Chemicals

Ethanol, 2-propanol, 1-propanol, *tert.*-butanol and sodium chloride (all analytical-reagent grade) were purchased from Merck (Darmstadt, Germany). Water was prepared with a Barnstead Nanopure Ultrapure Water System from International PBI (Milan, Italy).

2.2. Standards and spiked samples

The human albumin solutions in various concentrations from about 5 to about 20% (w/w) were obtained from Octapharma (Vienna, Austria).

For calibration purposes the final albumin solutions from the Cohn process, which always contain some level of ethanol, were lyophilized and redissolved in pure water to obtain ethanol-free standard solutions with 5% and 20% (w/w) albumin content by weighing in 5 g and 20 g per 100 ml, respectively. Spiked water samples as well as protein solutions were prepared by adding appropriate amounts of a stock standard solution containing 4 g ethanol/l. Thus spiked standard solutions in the concentration range of 10–100 μg ethanol/ml were obtained. In the same manner spiked solutions containing 10–100 μg ethanol/ml and 50 μg *n*-propanol/ml each were prepared for the internal standard experiments.

2.3. Instrumentation and conditions of analysis

Depending on the required volume, sample aliquots were transferred with either calibrated 20 μl capillaries or adjustable 5-ml Transferpettors (Brand, Wertheim/Main, Germany) into 22.5-ml headspace vials (Perkin-Elmer, Norwalk, CT, USA) which were sealed with a butyl septum and a crimp cap.

All headspace analyses were performed using a Perkin-Elmer HS 40 headspace sampler interfaced to a Hewlett-Packard Model 5890 Series II GC system (Hewlett-Packard, Wilmington, DE, USA) equipped with a split/splitless injection port and a flame

ionisation detection (FID) system. The column was a DB-WAX capillary 30 m×0.32 mm, 0.5 μm from J&W Scientific (Folsom, CA, USA). Data acquisition was carried out with a Hewlett-Packard Chemstation 3365. For further evaluation of the raw data and validation of the optimized process Microsoft-Excel (V 5.0), the Excel-macro ValiData (V 1.04, Rohrer/Wegscheider ASA, Graz, Austria) and Statgraphics Plus 3.0 (Manugistics, Rockville MD, USA) were used.

Helium 5.0 was used as carrier gas for headspace gas chromatography, the pressure on the headspace sampler was adjusted to 100 kPa. For conventional headspace analyses 2-ml sample aliquots were equilibrated for 10 min at a temperature of 60°C.

In case of the application of the full evaporation technique a sample volume of 20 μl was equilibrated at 100°C for 5 min.

The temperature of the transfer line and the injection port of the GC system was 150°C, the FID system was kept at a constant temperature of 250°C. The gas chromatograph was programmed to keep an initial temperature of 40°C for 8 min and to ramp at a rate of 15°C/min to a final temperature of 180°C which was held for 3 min.

2.4. Robust linear regression

This type of regression is preferably used when the values of the abscissa as well as the values of the *y*-axis are prone to error [14–16]. With the application of regular linear regression it is presumed that the numerical values of at least one variable be exactly known. A conventional calibration graph is obtained for example by plotting the concentrations of the analyte as precisely known values versus the signal intensities which contain the analytical errors. For linear robust regression, however, the erroneous analytical responses of two methods or of two series of measurements are compared against each other in order to disclose potential differences. As long as e.g., the signal intensities obtained from two different matrices are comparable within a predefined confidence interval of 95%, a linear graph with a slope=1 will result. A significant deviation of the calculated slope from the value of unity is indicative of the magnitude of the matrix effect.

2.5. Full evaporation technique

A brief definition of the constraints for the application of the full evaporation technique in static headspace analysis has been given in the Introduction. In the case of full evaporation of the volatiles they will be quantitatively transferred into the gas phase, which means, that the distribution coefficient $K \rightarrow 0$. Furthermore, the volume of the residual condensed phase becomes neglectible compared to the gas phase volume in the headspace vial. Thus, the well established sensitivity equation for static headspace analysis may be reduced to

$$C_g = M_0 / V_g \quad (1)$$

where C_g is the concentration of analyte in the gas phase, M_0 the original mass of analyte in the condensed phase and V_g the volume of the gas phase. Consequently, the concentration of the analyte is merely determined by the sample amount in the headspace vial. More detailed information on the theoretical background of static headspace analysis has been recently compiled by Kolb and Ettre [6].

3. Results and discussion

In order to document the effect of the protein matrix onto a direct static headspace analysis of ethanol, the results obtained from the analysis of simple aqueous samples and those obtained from aqueous protein samples were compared. For this purpose 2-ml aliquots of both matrices, distilled water and albumin solutions (20%, w/w) spiked with ethanol were analyzed and the peak areas of the corresponding peaks in the gas chromatograms were measured. For a characterization of the differences a robust regression of both sample types was calculated. Fig. 1 shows the result of the robust regression by comparing the peak areas of the gas chromatographic analysis of ethanol from the plain water matrix and from the aqueous albumin matrix. The peak areas determined from the standard matrix are assigned to the reference axis, those determined from the protein matrix are plotted on the other axis. The slope calculated from the experimental data is 0.9. This means, that the signal intensity of the ethanol

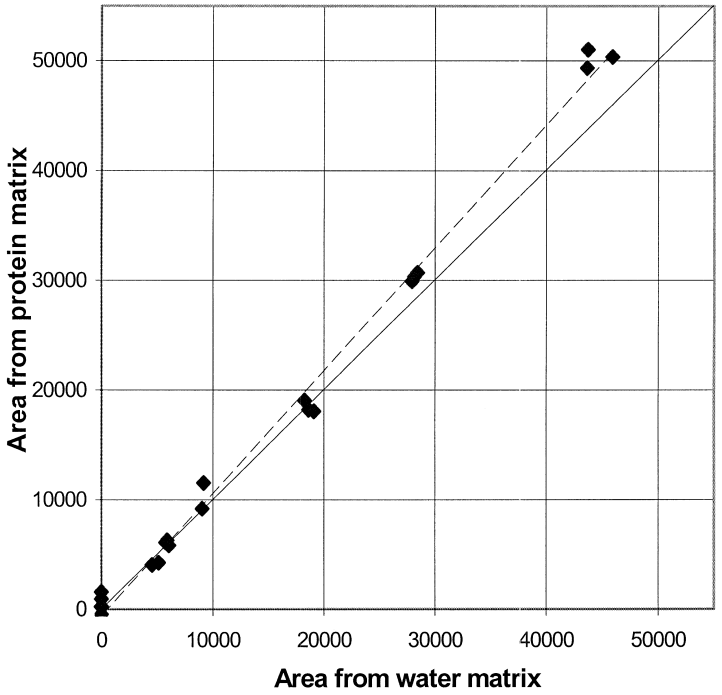

Fig. 1. Robust regression plot of the gas chromatographic peak areas of the ethanol peak for the estimation of the matrix effect. The solid line with a slope = 1 indicates ideal conditions with no interferences, the dashed line with a slope = 0.9 shows the experimentally determined influence of the sample matrix.

peak increases for about 10% with the analysis from the aqueous protein matrix compared to the pure water matrix. This observation corresponds with the data published by Hauck and Terfloth who reported enhanced ethanol responses at increased blood serum concentrations [17].

3.1. Internal standard calibration

In case of headspace gas chromatography several criteria have to be considered for the selection of a proper internal standard substance. Additionally to the basic requirement of high chemical similarity, the standard should elute in the chromatogram without overlap with sample signals, its retention time, however, should not differ too much from those of the analytes to be determined. Further concern has to be given to the response factor, volatility and the concentration of the reference compound which should be of the same order of magnitude compared to the sample components of interest. A more detailed experimental evaluation of the internal standard calibration for the estimation of volatiles has been presented by Drozd et al. [7].

In practice, alcohols like *tert.*-butanol, *n*-propanol and isopropanol can potentially meet those requirements and have been considered as internal standards for the analysis of ethanol in protein containing solutions [18,19]. The suitability of these substances as internal standards for the determination of ethanol in varying protein sample matrices was investigated, therefore. The two components isopropanol and *tert.*-butanol were less suited from the chromatographic standpoint, since either resolution or total time needed for analysis were beyond the desired limits under the given experimental conditions. *n*-Propanol proved to be best suited as an internal standard, since chromatographic separation from ethanol as well as analysis time were satisfying with the existing setup. Consequently, *n*-propanol was chosen for all further studies regarding the internal standardization procedure.

The experiments were carried out with the two sample matrices separately, deionized water and 20%

aqueous albumin solution containing ethanol and *n*-propanol as internal standard. The gas chromatographic headspace signals of the two alcohols from both matrix solutions were recorded and the resulting calibration graphs were computed by linear least-square regression. A direct means to estimate the systematic error of internal standardization is provided by a comparison of the slopes of the calibration graphs of the analyte normalized to the internal standard as they are obtained from different matrices. A significant influence of the sample matrix composition onto the distribution coefficient of analyte and of a potential internal standard component can be derived from the ratio of the individual slopes. The apparent calibration graphs for ethanol from a pure water matrix and a 20% aqueous protein matrix are shown in Fig. 2. It is obvious, that the responses of ethanol and *n*-propanol from the two sample matrices do not match. The numerical values of the slopes are 0.577 for the albumin matrix and 0.460 for the water matrix, resulting in a ratio of 1.25 which is indicative of the magnitude of the matrix effect. Apparently, the physico-chemical dissimilarity of the two alcohols is sufficient to have an impact on the distribution coefficients depending on the protein content of the liquid phase. In spite of the sterical similarity of both alcohols, calibration by internal standarization contributes inadequately to the overall attainable error in static headspace gas chromatography.

3.2. Full evaporation technique

The sample amount which can be processed for a single analysis depends on the size of the headspace vials and is restricted by the maximum volume that allows total vaporization of the volatile sample constituents. In practice, this amount may also become a limiting factor for the achievable sensitivity of this technique. Using the 22.5-ml standard

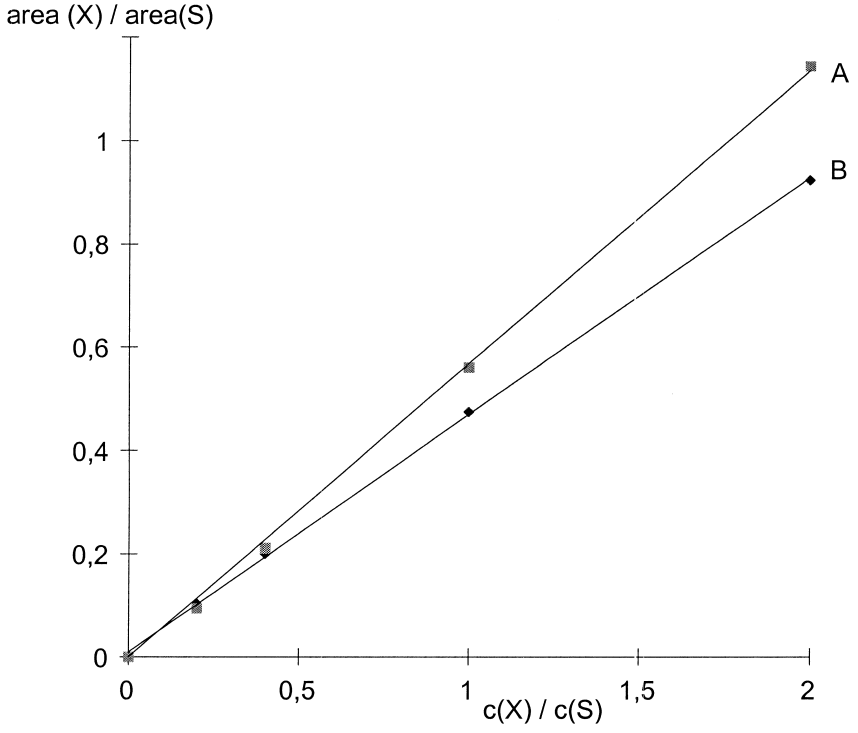

Fig. 2. Calibration graphs for the determination of ethanol using *n*-propanol as internal standard. (A) Aqueous protein matrix, (B) pure water matrix. Area (X)=peak area of ethanol, area (S)=peak area of internal standard, *c* (X)=concentration of ethanol, *c* (S)=concentration of internal standard.

headspace vials, the optimized experimental parameters were found to be 20 μl sample volume and 5 min of equilibration at 100°C. These conditions allow for a quantitative transfer of the analyte into the gas phase without noticeable sample decomposition. At higher temperatures and longer heating times several volatile decomposition products from the matrix were detected in the corresponding gas chromatograms, an oxidative formation of acetaldehyde from ethanol was not to be observed, however. All experiments were conducted with the chromatographic parameters adjusted to conditions as stated in Experimental. Spiked samples of both matrices, deionized water solutions as well as protein solutions, were analyzed and the resulting peak areas of the analyte were compared again by means of robust regression. Thus, the extent of a potential matrix effect is indicated by the corresponding slope of the curve.

From the robust regression calculation of the data a slope of 1.02 is obtained. The resulting regression graph is shown in Fig. 3. Computed from a data set of eight replicates at three concentration steps the limits of the slope are between 0.98 and 1.06 for a prefixed confidence level of 95%. Consequently, for the change of the protein content of an aqueous sample solution from 0% to 20% an influence of the matrix composition is not encountered. The application of this technique eliminates effectively the matrix effect and allows to calibrate even in plain aqueous solution. Fig. 4 shows a typical headspace chromatogram of a ''final container'' solution from the Cohn process. Ethanol elutes with a retention time of 5.925 min, interferences from other volatiles or degradation products are not to be observed.

According to the specified needs of quality control validation has been performed at four concentration levels in aqueous solution. All data were measured with four replicates and the resulting calibration graph has been evaluated. Mandel's test on linearity shows the suitability of the two-parameter model. The standard deviation of the method (s_{x0}) was computed from the quotient of the residual standard deviation and the slope of the regression graph. Its numerical value has been determined to be 3.7 mg/l.

The precision of the analytical system has been

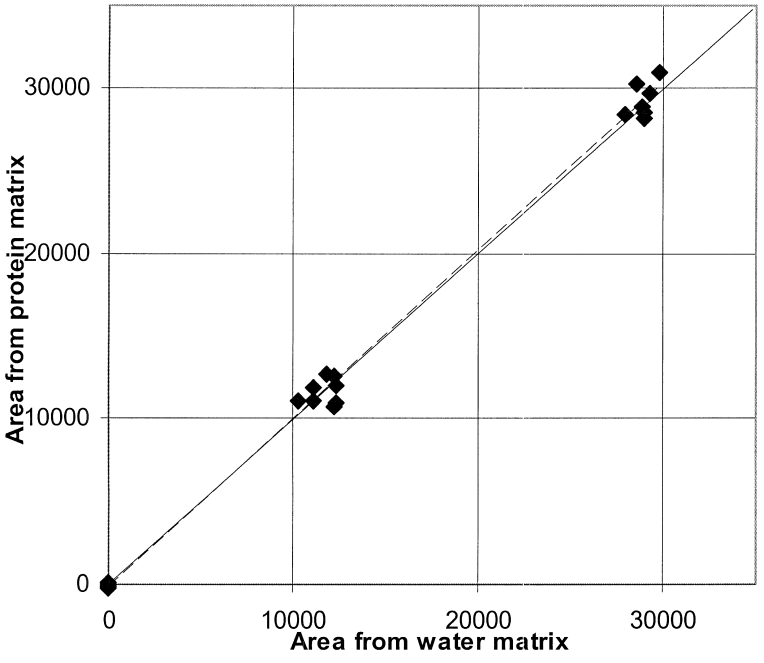

Fig. 3. Robust regression plot for the full evaporation technique. The nearly identical slopes from the experimental data (dashed line) and the ideal values (full line) indicate the elimination of matrix effects.

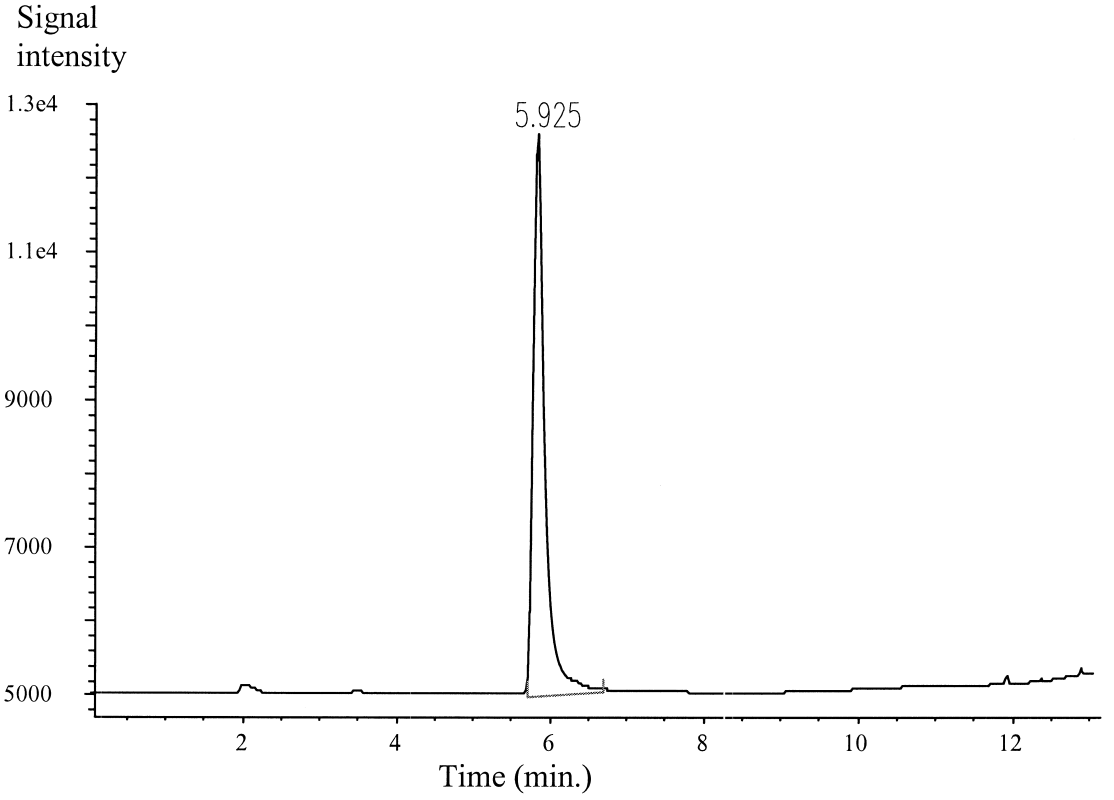

Fig. 4. Headspace gas chromatogram of 20 μl of a 20% (w/w) plasma protein sample from the Cohn process obtained with the full evaporation technique. The peak at 5.925 min corresponds to ethanol.

determined from the relative standard deviation of repetitive measurements of the same solution. Within-day repeatability was calculated from eight replicate measurements and is 2.9%, while the between-day repeatability (three consecutive days) equals 1%.

Signal linearity was proved with a correlation coefficient $r^2 = 0.997$ of the linear regression for the investigated concentration range. The limit of detection as calculated from the calibration graph [13] was 3.7 mg ethanol/l. This value is about 10-times higher than those obtained with conventional headspace techniques. This is mainly due to the smaller sample volume needed for the full evaporation technique, but it is still significantly lower than required for pharmaceutical quality control or even the analysis of so called non-alcoholic beer.

In order to check the robustness of the apparent method the total salt as well as the protein content of the liquid sample were varied within the specified limits. An alteration of the ethanol response was not to be observed for the range between 0–5 g NaCl/l and 0–20% (w/w) albumin concentration.

An additional test for the suitability of this method was performed by the analysis of an ''alcohol-free'' beer. This can be of common interest, since no standard validated method of analysis is presently available for the determination of low levels of alcohol in low-alcohol and no-alcohol beverages. The parameters of the analysis were chosen according to the optimized technique of full evaporation, special treatment or degassing of the sample was not necessary. The only precaution to be taken is, to transfer an exact aliquot of liquid sample without gas bubbles into the headspace vial. The analysis of several brands of ''alcohol-free'' beer revealed concentrations of ethanol in the range 0.2–0.5%. These results are in good agreement with the specifications of the producers and have not been verified by other

means. Further investigations with regard to this subject are in progress.

4. Conclusion

A method for a determination of ethanol in aqueous protein solutions with varying protein concentrations by means of combined headspace gas chromatography has been developed. The application of linear and robust regression analysis shows a significant influence of the protein content of the sample matrix onto the signal intensity of the ethanol peak. Apparently, the distribution coefficient of the analyte for the equilibrium between condensed phase and headspace above the liquid sample is shifted towards smaller values with increasing protein concentrations, resulting in an enhancement of the ethanol response. In order to provide for an universal calibration also for differently composed sample matrices, methods known to be potentially suitable for a reduction of the influence of the matrix composition were investigated.

Internal standardization was considered for matrix independent quantitation of the chromatographic signals. In general, structurally similar alcohols with comparable physical properties meet the requirements of an internal standard. For chromatographic reasons *n*-propanol was chosen in this study. A comparison of the influence of the matrix composition on the calibration curves of ethanol with *n*-propanol as internal standard exhibited a difference of the respective responses, which was not to be expected a priori.

Best results for a determination of ethanol in protein solutions by static headspace analysis could be obtained with the application of an optimized full evaporation technique. The theoretically expected and experimentally verified standardization of the distribution equilibria between condensed phase and gas phase eliminates effectively the interferences caused by the matrix composition. Thus, accurate external calibration may be accomplished with neat aqueous standard solutions for the analysis of liquid samples containing up to 20% (w/w) protein. Due to the small sample amounts to be used with this technique a reduction of sensitivity has to be taken into account, the detection limits, however, still being in the ppm range. All other performance characteristics are comparable or even superior to those obtained with conventional static headspace analysis. The standard deviation of the method, reproducibility, repeatability as well as method robustness are sufficient for the intended quality control purposes. The application of the full evaporation technique requires no further sample preparation steps, an addition of matrix modifiers or of standard solution to the sample is not necessary.

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