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Microdiffusion-based UV-LED spectrometric setup for determining low levels of ethanol in fruit juice

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ABSTRACT

A novel setup is described in which we combined the separation of a volatile substance from a sample with a complex matrix on the basis of a microdiffusion process with a kinetic on-line spectrometric monitoring of the reaction in the receptive medium at 365 nm. The fruit juice was selected as a model for testing the performance of the setup in real-life applications. The ethanol content in fruit juice can be considered as an indicator of the fruit-juice quality and should not exceed the regulatory limiting values. After optimising the microdiffusion process, blackcurrant, orange and two varieties of apple juice were analysed. The sample analysis lasted 15 min at 35 °C. The ethanol concentrations were found to be between 0.9 and 4.0 mmol/L, and were comparable to the results obtained using the SIST:ISO 2448:1998 standard method, which is time consuming, labour intensive and requires high sample volumes. The setup can easily be adapted for determining other volatile substances in low concentrations in complicated samples of different types by introducing different chemistry and replacing the light source if the light of a different wavelength is required. The measuring characteristics of the setup were critically assessed, the main sources of uncertainty recognised and the possibilities for further improvements of the setup and the procedure considered.

1. Introduction

Constituents present in low quantities in a complex matrix still represent a challenge for chemical analysis. Clinical, forensic and food samples are examples for which a determination of ethanol in low concentrations can be of great interest.

Fruit juices should, preferably, not contain any ethanol; however, frequently some ethanol is present in small quantities due to the fruit-fermentation processes. In an earlier study, for non-treated, glass-packed, grapefruit sections it was shown that ethanol can be considered as an indicator of spoilage [1]. The acetaldehyde and ethanol levels in litchi fruit may be considered as predictors of over-ripeness [2]. The occurrence of off-flavours which mandarins develop is related to the increases in juice ethanol and the levels of acetaldehyde [3]. The head-space, GC method with a flame-ionisation detector was used in these studies.

Two different upper limits for the ethanol content in fruit juices can be found in the legislation: either 0.5%, expressed as a volume fraction of ethanol, or 3 g/L, expressed as the ethanol mass concentration. The concentrations of ethanol in real samples are usually much lower.

Even though the GC method is the method of choice when more detailed information on the volatiles present in a fruit juice is required, this method is not widely recognised as an appropriate standard method, e.g. the prEN 1369 standard method for determining ethanol content in fruit and vegetable juices with GC, which was published in 1999 [4] was withdrawn in 2003.

Among other methods for determining ethanol in low concentrations, electrochemical sensors or biosensors, mostly amperometric, strongly prevail [5–13]. Even though the reported concentration ranges of the methods are frequently appropriate for determining the ethanol content in fruit juices, the usability of the sensors for real-life applications was only demonstrated on examples of alcoholic beverages. Among the UV-LED based devices a fibre optic bio-sniffer [14] and biosensor [15] were reported for ethanol determination.

The SIST:ISO 2448:1998 distillation–retitration standard method ($K_2Cr_2O_7$, $(NH_4)_2Fe(SO_4)_2$) is widely accepted as a way of determining the ethanol content in fruit and vegetable products [16]. It is intended for samples not containing more than 5% of ethanol. The method is labour intensive and time consuming and requires more than a half of a litre of a fruit juice sample. It gives more an insight into an important aspect of the quality of a fruit juice than presents the ethanol content only since some other

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volatile substances, e.g. methanol and acetaldehyde contribute to the final result. Consequently the methods involving similar chemistry can offer an alternative.

Three FIA methods [17–19] employing the same reaction as the standard method for determining ethanol in concentrations characteristic for alcoholic beverages were described in the literature. The limits of detection are too high for the fruit-juice analyses.

In 1939 Conway published a book [20] in which he described a microdiffusion dish, which is called after him, and suggested several procedures for microdiffusion analyses of volatile compounds in low concentrations in samples with a complex matrix, mainly clinical or forensic. The external compartment in a form of a ring accepts a sample containing a volatile substance under consideration; the internal compartment is intended for a receptive medium in which the volatile substance accumulates and reacts after travelling the distance between the two compartments through the gas phase in a closed system by a microdiffusion process. Apart from the microdiffusion method for the blood ammonium which reached the widest popularity, Conway among many other methods also described a 2-h microdifusion procedure for determining ethanol in urine or blood and a 3-h method for determining ethanol in tissue, both followed by an iodometric titration.

Many decades later some advantages of the microdiffusion are still recognised and microdiffusion is used for example: as a preanalytical step for determining the N-15 isotope ration in soil samples [21–24] for determining the cyanogenic glycoside in human urine [25], total cyanogens in fresh cassava [26] and cassava flour [27], fluoride in milk [28] and soil and plant materials [29]. With the described methods the microdiffusion lasted from several hours to even days. Even a procedure for determining cyanide and azide in beverages which was declared as a rapid method [30] and was performed at 40 °C lasted an hour. A spectrometric method was used after the microdiffusion process was accomplished.

No setup is known to the author that enables the on-line monitoring of the microdiffusion process in a Conway dish or other microdiffusion chamber taking advantage of the simplicity of this separation and preconcentration approach requiring low sample volumes and combining it with a kinetic method for obtaining useful results for real samples in 15 min.

The objective of this research was to develop a setup that would be sensitive enough to enable a determination of the ethanol concentration in fruit juices in a shorter time, with lower sample volumes and with a lower energy consumption than the standard method, and would also have the potential to be developed into an instrument for at-line monitoring or field testing of other volatile substances in complex matrix.

2. Experimental

2.1. Chemicals

The sulphuric acid, H_2SO_4, w Y = 96 ± 1%, ρ (20 °C) = 1.835 g/mL, purchased from Carlo Erba, Italy; the potassium dichromate, $K_2Cr_2O_7$, $w \geq 99.5\%$, purchased from Lapharma, Skopje, Bosnia; the Triton X-100, $C_{33}H_{66}O_{10.5}$, M Y = 646.37 g/mol, chromatography grade, purchased from Merck, Germany, and ethanol C_2H_5OH, w = 96.1–96.9%, ρ = 0.81 g/mL, purchased from Scharlau, Spain were used for the preparation of the reagents and standards. All the solutions were prepared with deionised water that was additionally purified through a Milli-Q system (Millipore).

2.2. Preparation of solutions

The sulphuric acid solution (1 + 1) was prepared by combining equal volumes of the sulphuric acid and water and used for the preparation of the standard potassium dichromate solution 3.40 mmol/L, for which the $K_2Cr_2O_7$ was dried for 2 h at 105–110 °C. The standard solutions of ethanol with the concentrations 1.029, 2.058, 3.087, 4.115 and 5.144 mmol/L were prepared. The Triton X-100 solution, 1 mmol/L, was prepared and used as a (1 + 1) diluent for lowering the surface tension of all the examined solutions prior to them being transferred into the external chamber of the Conway microdiffusion dish.

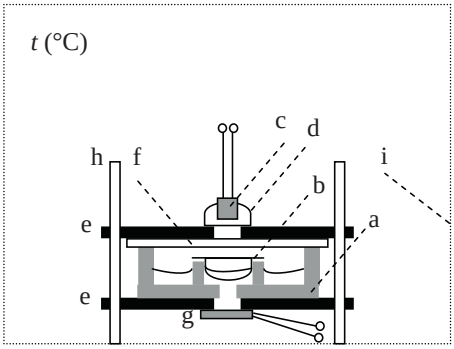

Fig. 1. Microdiffusion-based UV-LED spectrometric setup: (a) Conway microdiffusion dish, (b) disposable polymeric spectrometric cell, (c) UV LED, (d) LED reflector, (e) support, (f) glass lid, (g) photoresistor, (h) stand, (i) metallic compartment.

2.3. Microdiffusion-based UV-LED spectrometric setup

The microdiffusion-based UV-LED spectrometric setup is schematically presented in Fig. 1. We drilled a hole (Φ 3.3 mm) through the centre of the bottom of the ceramic Conway dish (Thomas Scientific, USA) (a). With our previous research we confirmed that the hollows of the polymeric supports, called blisters, can function as disposable spectrometric measuring cells [31,32]. The edges around the hollows were cut in the shape of a circle so that the spectrometric cell (b) seats tightly in the central compartment of the Conway dish. The UV LED, λ_{max} = 365 nm, Nichia (c) was inserted into a LED reflector (d) and mounted on the support (e) that was positioned on the top of the glass lid (f). The photoresistor (Delko, Slovenia), (g) was positioned into the optical path. The components were assembled and mounted into a stand (h). The setup was inserted into an in-house-made metallic compartment (i) that was immersed into a UC-8A thermostat bath (Julabo, U.S.A.). The UV LED and the photoresistor were wired as described previously [31]. An M-3860 digital multimeter (Metex Corporation, Korea) with a serial output and the accompanying MultiView software was used as an interface to the computer and as a data-acquisition device. The absorbance values were calculated from the transmittance measurements in a MS Excel spreadsheet. For the statistical evaluation of the results the Statgraphics Centurion, version XV software (Timberlake, Herndon, Virginia) was used.

2.4. Microdiffusion experiments

For a microdiffusion experiment 210 μL of the solution of the sulphuric acid (1 + 1) were transferred into the spectrometric cell, which was inserted into the central compartment of the Conway microdiffusion dish. The setup was assembled and the transmittance adjusted to 100.0%. Afterwards the spectrometric cell was replaced by a spectrometric cell containing 210 μL of the 3.40 mmol/L standard potassium dichromate solution in the sulphuric acid (1 + 1) as the receptive medium for a microdiffusion experiment. A 1-mL test portion of the solution obtained previously by mixing the Triton X-100 solution (1 mmol/L) with a blank or a standard ethanol solution or a model solution or a

sample in the volume proportion 1 + 1 was transferred into the external compartment of the Conway microdiffusion dish. The Conway dish was immediately closed tightly using a lid with a silicon-grease seal and the whole setup was reassembled. The UV LED was switched on simultaneously with the initiation of the computerised data acquisition. Measurements were collected at 10-s intervals for 60 min. The temperature inside the compartment was recorded periodically during the whole run.

2.5. Standard method

The only deviation from the standard method [16] was in a fruit juice sample size (700 g).

3. Results and discussion

3.1. Optimisation of the composition of the potassium dichromate solution

For optimising the composition of the potassium dichromate solution for the internal compartment of the Conway microdiffusion dish the measuring characteristics of the setup were evaluated to find out the measuring rang in which the absorbance measurements are the most reliable. For evaluating the repeatability of the measurements between runs at different absorbance values we were looking for a stable absorption medium. The polymeric filter foils Lee Filters 003, 004, 102, 008 and 100 (Andover, England) were determined to be the most appropriate and selected because of their absorption characteristics around 365 nm. The measuring system exhibits percentage standard deviation between 1 and 2% ($n = 8$), for the absorbance measurement in the range between 0.7 and 0.3, taking into account the uncertainty introduced into the procedure by disassembling and reassembling the setup after each measurement. The percent standard deviation already raised to 4.1% at the absorbance 0.198. For this reason the absorbance measurements below 0.3 are less favourable and the range above this value was recognised as the target range for the microdiffusion procedure during its optimisation. It was confirmed that the absorbance values (0.167–0.971) are linearly related to the number of overlaid layers of the 103-Lee Filters filter foils with the correlation coefficient $R^2 = 0.9979$.

Different volumes and concentrations of the standard potassium dichromate as the ethanol-receptive medium in the spectrometric cell in the central compartment of the Conway microdiffusion dish were examined afterwards. The 210 μL volume and the 3.40 mmol/L concentration of the standard potassium dichromate solution were recognised as the optimal choice with the absorbance in the target range, at the beginning of a run as well as during the kinetics of the reaction between ethanol and dichromate.

3.2. Optimisation of the microdiffusion experiment

The microdiffusion experiments were afterwards performed with different ethanol concentrations namely 1.029, 2.058, 3.087, 4.115 and 5.144 mmol/L, as described in Section 2.4. The whole set of experiments was repeated at the temperatures 25 °C, 30 °C, 35 °C, 40 °C and 45 °C. The microdiffusion process was, during each experiment, followed by acquiring the transmittance over time with a measurement-acquisition frequency of one measurement per 10 s. The transmittances were recalculated into absorbances and the curves of the absorbance dependence with time were further analysed. The curves obtained at 25 °C for the microdiffusion experiments with the standard ethanol solutions with concentrations 0, 1.029, 2.058 and 3.087 mmol/L are presented in Fig. 2. The steepest curve corresponds to the 3.087 mmol/L ethanol concentration. The curves obtained for the highest ethanol concentrations, i.e. 4.115 and 5.144 mmol/L, were omitted from the graph for the sake of clarity. Similar graphs were obtained for all the other temperatures.

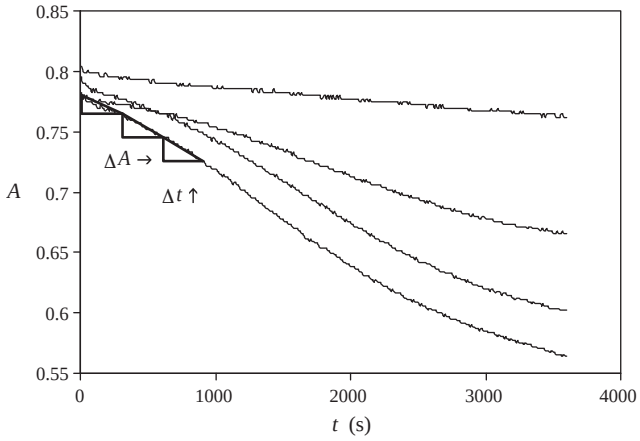

Fig. 2. The curves of the absorbance dependence on time obtained by following the reaction of ethanol with potassium dichromate during the microdiffusion process in the Conway microdiffusion dish for the experiments with the standard solutions with ethanol concentrations of 0, 1.029, 2.058 and 3.087 mmol/L, respectively (top to bottom).

The curves were analysed so that different time intervals of different length, either successive or partially overlapping, were taken for calculating the first derivatives ($\Delta A/\Delta t$). Among the various tested scenarios for analysing the curves the quotients $\Delta A/\Delta t$ for the successive 5-min time intervals were recognised as an optimal choice and among them $\Delta A/\Delta t$ for the third 5-min time interval (610–910 s) indicated in Fig. 2 exhibits the strongest linear correlation with ethanol concentration at all temperatures as presented in Fig. 3. In Fig. 3 the regression lines obtained at 25 °C, 30 °C, 35 °C, 40 °C and 45 °C are compared. The lines follow from the top to the bottom in the same sequence as the temperatures were listed. The comparison of the correlation coefficients confirmed the strongest correlation for the line in bold obtained at 35 °C.

The microdiffusion-based UV-LED spectrometric setup proved useful for a quantitative determination of the ethanol in low concentrations. The study in the continuation focussed on its usability in real-life applications.

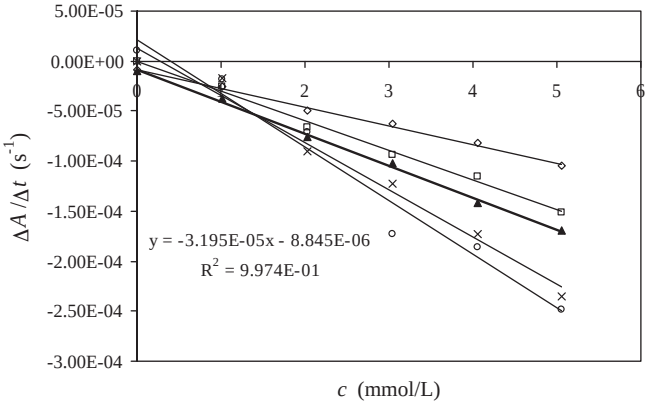

Fig. 3. Correlation between the first derivative $\Delta A/\Delta t$ (time interval 610–910 s) and ethanol concentration obtained at different temperatures (25, 30, 35, 40 and 45 °C, top to bottom).

Table 1
Determination of ethanol concentration in model ethanol solutions with saccharose concentrations of 130 g/L or 65 g/L (*).

$\Delta A/\Delta t$ (s^{-1})	c_{obt} (mmol/L)	$U(c_{obt})$ (mmol/L)	c_{exp} (mmol/L)	c_{obt}/c_{exp}
-3.736×10^{-5}	0.89	±0.25	1.013	0.88
-4.400×10^{-5} (*)	1.10	±0.25	1.013	1.09
-7.521×10^{-5}	2.08	±0.24	2.025	1.03
-6.394×10^{-5} (*)	1.72	±0.24	2.025	0.85
-1.086×10^{-4}	3.12	±0.24	3.038	1.03
-9.568×10^{-5} (*)	2.72	±0.24	3.038	0.89
-1.628×10^{-4}	4.82	±0.26	4.051	1.19
-1.184×10^{-4} (*)	3.43	±0.24	4.051	0.85

Table 2
Determination of ethanol in fruit juices.

Sample	$\Delta A/\Delta t$ (s^{-1})	c_{obt} (mmol/L)	$c_{titration}$ (mmol/kg)
Blackcurrant juice	-1.363×10^{-4}	3.99	3.88
Apple juice 1[a]	-6.199×10^{-5}	1.66	1.82
Apple juice 1[b]	-6.690×10^{-5}	1.82	1.67
Orange juice[a]	-5.171×10^{-5}	1.34	1.53
Orange juice[b]	-4.842×10^{-5}	1.24	1.58
Apple juice 2[a]	-4.078×10^{-5}	1.00	0.93
Apple juice 2[b]	-3.887×10^{-5}	0.94	1.08

3.3. Performance in real-life applications

The fruit juice was selected as a model for testing the performance of the setup in real-life applications.

In order to test if a matrix changing the osmolarity of the tested solution affects the transfer of ethanol in the Conway microdiffusion dish the experiments with different concentrations of ethanol were repeated at 35 °C with the standard ethanol solutions prepared in the saccharose matrix with a saccharose mass concentration 130 g/L. The equation of the regression line obtained for the saccharose matrix and the equation of the line obtained previously at 35 °C with no matrix (Fig. 3 line in bold) were compared; and at the 90% or higher confidence level no statistically significant difference, neither between the intercepts ($a_{saccharose} = -9.6 \times 10^{-6}$, $a = -8.8 \times 10^{-6}$, $s_{a\_saccharose} = \pm 9.6 \times 10^{-6}$, $s_a = \pm 2.5 \times 10^{-6}$) nor between the slopes ($b_{saccharose} = -3.26 \times 10^{-5}$, $b = -3.19 \times 10^{-5}$, $s_{b\_saccharose} = \pm 3.2 \times 10^{-6}$, $s_b = \pm 8.2 \times 10^{-7}$) of the two lines, was confirmed, leading to the conclusion that the saccharose matrix does not affect the transfer of ethanol in the Conwy microdiffusion dish.

The model solutions with different known concentrations of ethanol and saccharose in mass concentrations of 130 g/L or 65 g/L were analysed next with a microdiffusion at 35 °C. The ethanol content in these solutions was derived from the interpolation of the calibration line for ethanol with no added saccharose (c_{obt}). The expanded standard uncertainties for the interpolation of the results from the regression line $U(c_{obt})$ were calculated with the coverage factor $k = 2$. The results, which are summarised in Table 1, were compared to what was theoretically expected (c_{exp}) and the recoveries c_{obt}/c_{exp} were calculated. The values are between 0.85 and 1.19.

The fruit juices were analysed in the same way as the model solutions. The ethanol concentration was, for a comparison, also determined using the standard method (SIST ISO 2448:1998) ($c_{titration}$). The results in Table 2 confirmed that the microdiffusion-based UV-LED spectrometric setup was useful for differentiating among the different levels of ethanol in real samples with a complex matrix. The superscript a or b indicates that the same juice variety with the same lot number (but from two different packs) was analysed. The highest concentration of ethanol was determined in the blackcurrant juice and the lowest in the apple juice 2. The concentration levels determined by the two methods in real samples were comparable taking into account the expanded standard uncertainties from the Table 1. The result for orange juice (1.24 ± 0.25) mmol/L is lower than the result obtained with the standard method.

In the continuation the main sources of uncertainty that might have affected the results are critically assessed and further possibilities for improvements to the prototype and the procedure are suggested.

3.4. The main sources of uncertainty

3.4.1. Geometric parameters and measuring characteristics of the setup

The setup in its present form requires the assembly of several parts before each run. The positions are not strictly defined but mainly controlled by visually inspecting some key position markers or indicators. The introductory step during which the intensity of the light source is regulated and the absorbance of the filter foil is measured was an overall control procedure before each run. In order to account for the importance of these influences the absorbance of the 100 Spring Yellow filter foil was measured in the Conway microdiffusion dish for 60 min and the procedure was repeated six times. The $\Delta A/\Delta t$ values were calculated for the 610–910 s interval; in four cases the value was 0 and in the remaining two the values were -6.717×10^{-06} and -6.943×10^{-06}, respectively. The range of the $\Delta A/\Delta t$ variability is of the same magnitude as the standard deviation of the intercept of the calibration line obtained at 35 °C. The geometric parameters and the measuring characteristics of the setup cannot be considered as a significant source of uncertainty, especially because in four cases out of six no influence at all on the $\Delta A/\Delta t$ was observed.

3.4.2. Transfer of water in the Conway microdiffusion dish

The solution of the potassium dichromate contains sulphuric acid in a high concentration hence not only ethanol but also water is transferred from the tested solution in the external compartment of the Conway microdiffusion dish into the potassium dichromate receptive solution in the spectrometric cell in the central compartment.

As Fig. 4 demonstrates the temperature was recognised as the main factor influencing the water transfer (Δm). The dotted lines represent the water transfer from the solutions with different concentrations of ethanol prepared with no added saccharose. The solid or bold lines correspond to the solutions containing saccharose in concentration 65 or 130 g/L, respectively. The average increases of the masses of the spectrometric cells during the 60-min microdiffusion experiments at 25 °C, 30 °C, 35 °C, 40 °C and 45 °C were 24.9 mg, 31.0 mg, 37.8 mg, 47.1 mg and 57.6 mg, respectively. No statistically significant difference between the means of the three series was confirmed at a 95.0% confidence level for 25 °C and 30 °C. In contrast, a statistically significant difference between the means of the series with no saccharose and the saccharose concentration of 130 g/L was confirmed at a 95.0% confidence level for all the other temperatures; and for the highest two, 40 °C and 45 °C, a statistically significant difference between the means of the series with

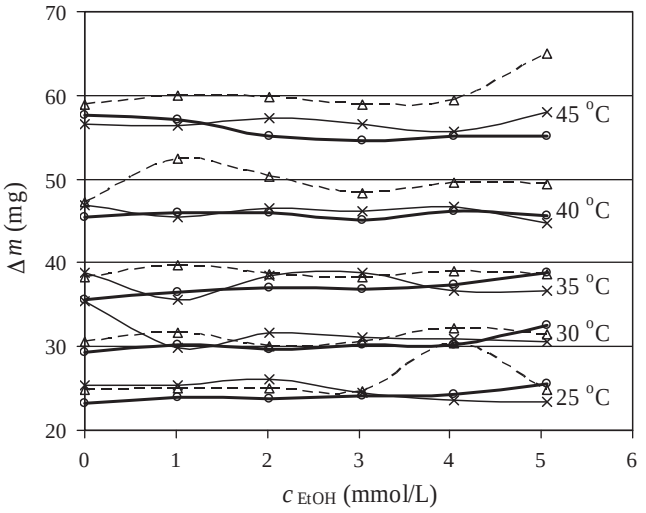

Fig. 4. Transfer of water in the Conway microdiffusion dish at different temperatures, for the standard solutions of ethanol with different matrix (no saccharose – dashed line; saccharose concentration 65 g/L – solid line; saccharose concentration 130 g/L – bold line) measured as a difference in the mass of the standard potassium dichromate solution (Δm) in the spectrometric cell before and after a microdiffusion experiment.

no saccharose and the saccharose concentration of 65 g/L was also confirmed.

Consequently it was important to find out if the absorbance measurement can be significantly affected by the water transfer during a microdiffusion run. The absorbance of the standard potassium dichromate solution (210 μL) in the spectrometric cell was measured six times, with the setup being disassembled and reassembled each time. Afterwards, 40 μL of deionised water were added to the standard potassium dichromate solution simulating the expected water transfer at 35 °C and the measurements were repeated. The whole experiment was repeated with a 60-μL addition of deionised water simulating the expected water transfer at 45 °C. The mean absorbance values and standard deviations obtained during the two phases of these two experiments were 0.829 ± 0.014, 0.826 ± 0.013, 0.836 ± 0.018 and 0.811 ± 0.017 and no statistically significant differences between the four means were confirmed at a 95.0% confidence level. The explanation of these observations lays in the vertical optical geometry of the setup.

3.4.3. Thermal stability during the runs and sample manipulation

The highest thermal instability was observed during the first 10 min. The temperature variations in the compartment are in the range ±1 °C at most and are much higher than expected from the temperature accuracy of the thermostat (±0.2 °C). The reason is probably that the compartment partially blocked the circulation of water in the bath. Even though the time interval between 10 and 15 min turned out to be the interval of relatively good thermal stability the sample or calibration solutions' manipulation and their introduction into the Conway microdiffusion dish and the thermal stability of the whole system can be seen as the weakest points of the setupsetup and the procedure in their present form and a further improvement would be advisable.

3.4.4. Calibration

For practical reasons the experiments lasting 60 min were carried out in such a way that the microdiffusion with a single calibration solution was studied at all temperatures on the same day. Consequently, the points of the same calibration line originated from the experiments performed on 6 separate days and experiments with the model solutions and the fruit-juice samples were performed on other separate days.

With the final optimised microdiffusion procedure, which lasts only 15 min, the calibration and the sample analyses can be performed in a series on the same day and by replicate measurements.

4. Conclusions

This research confirmed that the microdiffusion-based UV-LED spectrometric setup is useful for determining low levels of ethanol in a complex matrix. The meaningful results for fruit juices were obtained in 15 min from 1-ml sample volumes at 35 °C, in contrast to the standard titration method, for which 700 g of sample are required and the sample has to be distilled for approximately 1 h. Furthermore, the final result cannot be obtained in much less than 2 h. For a higher throughput several similar units can be produced at a low-cost or the setup can be designed as a multichannel system in which the same electronic unit acquires data from several Conway microdiffusion dishes simultaneously. The usability of the setup is not limited only to this application. The setup can easily be adapted for determining other volatile substances in complicated samples of different types by introducing different chemistry and replacing the light source if the light of a different wavelength is required.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.09.058.

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