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ARTICLE

Application of gas-diffusion microextraction to solid samples using the chromatographic determination of α-diketones in bread as a case study*

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Gas-diffusion microextraction (GDME) was applied in the extraction of vicinal diketones in bread samples aiming the determination of these compounds by high-performance liquid chromatography with UV-Vis detection (HPLC-UV). For the first time, GDME was used for direct chemical determination in solid samples, i.e. avoiding any sample preparation prior to extraction. Different extraction parameters were studied and optimized, namely temperature, time and chemical composition of the acceptor solution where o-phenylenediamine was used as a derivatizing agent, originating quinoxalines that could be determined at 315 nm. GDME demonstrated to be a good tool for the sampling of volatile compounds in solid samples with suitable method features for butane-2,3-dione (diacetyl, DC), pentane-2,3-dione (PN) and hexane-2,3-dione (HX): low LODs (6.0, 8.6 and 12 μg kg^{-1}, for DC, PN and HX respectively) and LOQs (20, 29 and 38 μg kg^{-1}, for DC, PN and HX respectively), r^2 above 0.990, and CV around 5%. The developed methodology was applied in the determination of different bread samples and was used to access the decrease of α-diketones in bread during a week timeframe.

Introduction

Although many of the samples that are analysed in a regular basis are solid, ranging from sources like food products passing by tissues of living organisms to industrial products,[1] and many more, the direct analysis without any pre-treatment of the solid samples still remains challenging in analytical chemistry. The regular process normally requires some form of extraction, in many cases with an organic solvent, and some kind of physical process like agitation or sonication; these processes not only are costly in terms of equipment, solvents and time but also not very environmentally friendly.

Gas-diffusion microextraction (GDME) is a technique which initial development occurred just a few years ago.[2, 3] It comprises the advantages of microextraction with typical membrane-aided gas-diffusion techniques, and has been used for several different analytes in different samples using various detection techniques.[4-7] It is particularly advantageous when combined with a derivatizing reaction taking place in an acceptor solution. A small amount of the analytes filling the sampling chamber pass thorough the probe's membrane, and are chemically trapped by the derivatizing reaction. Recently, it has been demonstrated the GDME's applicability in aiding the determination of low volatile compounds, like methylglyoxal,[4] and in semi-qualitative analysis.[5]

Only a few membrane-based analytical approaches, where solid samples are directly analysed without being previously dissolved nor having had any substantial sample manipulation, can be found in literature. Early examples include the determination of ammonium in food samples[8] and in soil with spectrophotometric[9] or potentiometric detection;[10] and a microwave-assisted system for the determination of mercury in solid samples.[11]

Herein, GDME was applied in the determination of vicinal diketones in bread. Vicinal diketones are a relevant class of compounds in several food matrices including milk, dairy products, coffee, popcorns, wine and beer.[6, 12, 13] Among this chemical group, diacetyl (DC, butane-2,3-dione) plays a more significant role. DC is a potent key aroma in bread, that is produced both in a Maillard reaction as well as generated by thermal degradation of carbohydrates during baking at relatively higher temperatures, particularly the crust, being also produced during toasting.[14-16] Its determination is relevant not just for the final product quality control but also during the

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* Dedicated to Professor Aquiles Araújo Barros on the occasion of his 65th birthday

brewing process as a control parameter. However, α-dicarbonyls are also relevant due to health related concerns: continuous DC exposure has been associated to pulmonary diseases,[17] Alzheimer's disease and cancer.[18]

Experimental

Chemicals and samples

All reagents used were of analytical grade and were used without further purification. Ultrapure water (resistivity not less than 18.2 Ω cm at 298 K) from a Direct-Q 3UV water purification system (Millipore) was used in all experiments.

Solutions of DC, pentane-2,3-dione, (PN) and hexane-2,3-dione (HX) of concentration *ca.* 1.00 x 10^{-3} mol/L, were prepared daily from stock solutions of each vicinal diketone (*ca.* 0.100 mol/L). The stock solutions were monthly prepared in ultrapure water and stored in the fridge at 4 °C. All vicinal diketones, sodium chloride, sodium acetate and sodium carbonate were purchased from Sigma-Aldrich. Phosphate buffer 0.1 mol/L, pH 7.0, was prepared using disodium hydrogen phosphate (Merck) being the pH adjusted with 1.0 mol/L hydrochloric acid (Merck). The 0.1% m/m *o*-phenylenediamine (OPDA, Merck) in phosphate buffer solution was prepared daily and kept in the dark.

Bread samples were purchased in local supermarkets and bakeries. Prior to the extraction bread was grinded using a commercial food chopper with a sieve size of 0.25-2.00 mm (Retsch AS 200).

Chromatographic analysis

The HPLC system (Jasco Corporation) consisted of a quaternary low-pressure gradient pump (model PU-2089 plus) with an in-line DG-1580-54 degasser, a Rheodyne 7725(i) sample injection valve and a LC-Net II/ADC interface with a computer. The system was equipped with a Jasco MD-1510 UV/Vis spectrophotometric detector. Control and data analysis were executed with a Jasco ChromPass Chromatography Data Software version 1.7.403.1.

Chromatographic separation was performed in a column Phenomenex Gemini C_{18} (250 x 4.60 mm, 5 µm), in an isocratic mode 50% acetonitrile and 50 % acetate buffer (0.01 mol/L, pH 4) at 0.8 mL/min, during 20 minutes, with UV detection at 315 nm. 100 µL of sample was injected into the chromatographic column kept at room temperature. Analytes were identified by their retention time and by means of the injection of standards. All chromatographic eluents were filtered through a Nylon filter of 0.45 µm pore size (Whatman) prior to use.

Extraction procedure

The basic extraction principles of GDME have been previously described,[2] nevertheless minor modifications were performed in order to adapt it to solid samples. The thermostatized chamber (Metrohm, titration vessel with thermostat jacket, ref. 6.1418.220) where the sample was placed had a volume of *ca.* 30 mL. A scheme of the extraction is shown in Figure 1, the lid sealed the chamber by means of an o-ring, also using an o-ring the GDME probe was attached to the lid creating a closed environment.

Analytes were extracted from the sample by a gas-diffusion process through a gas-permeable hydrophobic membrane (Millipore Mitex 5.0 µm) to an acceptor solution containing the derivatization reagent, OPDA.

Except when mentioned otherwise, the following procedure was used: a) 5 g of grinded bread were placed inside the thermostatized sampling chamber; b) extraction occurred at 65 °C during 15 minutes; c) the acceptor solution consisted of 500 µL of 0.1% OPDA in phosphate buffer; d) an aliquot of the extract was analysed by HPLC-UV according to the already mentioned chromatographic procedure. When required, standard additions were performed by adding small volumes of standard solutions directly to the solid samples.

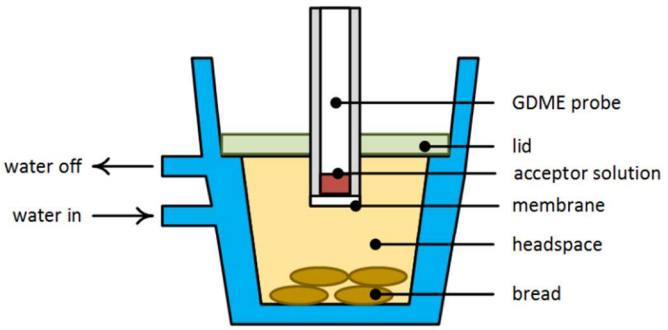

Figure 1 – Extracting scheme. The bread sample was placed inside a thermostatic chamber, a doubled wall glass vessel whose temperature was controlled by a circulating water bath, a poly(methyl methacrylate) lid seals the headspace; the GDME probe with a hydrophobic membrane containing a small volume of acceptor solution is suspended inside.

Results and discussion

Acceptor solution

Since vicinal diketones are not directly determined by UV-spectrophotometry, a derivatizing reaction was required. The derivatizing reaction was based on the classical Hinsberg reaction,[19] the two OPDA's amino groups connect to the other compounds by the two carbonyl groups forming a second ring, i.e. quinoxalines derivatives. Quinoxalines can be easily sensed by spectrophotometry, fluorometry or electrochemistry. This derivatizing reaction can be used for a wide range of compounds, including dehydroascorbic acid,[20] epinephrine,[21] amino acids,[22] pyruvic acid,[23] glyoxal[24] and, of course, other α-dicarbonyls[25, 26] including DC[6, 27] in also a large number of different samples. The study of this derivatizing reaction, including parameters like temperature, time and pH, is described elsewhere.[24]

Microextraction advantages include procedure simplification, reduced time associated with conventional sample clean-up and, notoriously, analyte enrichment.[28] This analyte enrichment, for the same extracting time and before an exhaustive extraction, is greater when the ratio sample/extract is augmented. In Figure 2, the minimal volume tested (300 µL)

originated the larger chromatographic peak area. The volume was not decreased further due to practical issues, smaller volumes up to drop size would need a different design of the GDME probe and the use of micropipettes of smaller volumes or microsyringes and even a certain degree of automation,[29] for the time being these technicalities were not addressed. Additionally, one has to guarantee an adequate extract volume for a suitable liquid chromatographic separation.

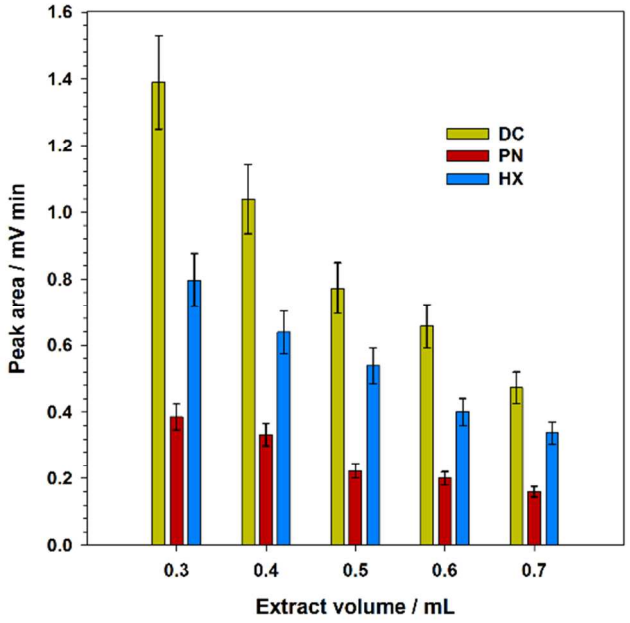

Figure 2 – Chromatographic peak area variation with the volume of extracting solution, five different volumes were tested from 300 to 700 µL, extractions were performed on 5 g of a bread sample with an aliquot of 100 µL of a solution with 15 mg L^{-1} of DC, PN and HX.

Temperature of extraction

Considering that it is being discussed a diffusional process, obviously the temperature of extraction is an experimental parameter that should be carefully controlled. Results obtained for a range of temperatures from 25 to 65 °C are displayed in Figure 3 (higher temperatures may lead to evaporation of small volumes of the extracting solution). As expected, the analytical response increases with an increasing temperature of extraction. From a theoretical point of view, and assuming the system is in equilibrium, this could be explained by the increase in the partial pressure of the analytes according with the Clausius-Clapeyron relation:[30]

$$\frac{\partial P}{\partial T} = \frac{L}{T\Delta v}$$

where P is the pressure, T is the temperature, L is the specific latent heat and Δv is the specific volume change of the phase transition. Since Δv can be approached to RT/P, where R is the specific gas constant. Thus, the initial equation can be simplified in this case to:

$$ln\, P = \frac{a}{T} + b$$

where a and b are curve fitting parameters and T is the absolute temperature. Considering that the partial pressure is related to the peak area obtained in the HPLC-UV analysis, the logarithm of peak area vs. the inverse of temperature could be plotted linearly as shown in the inlay of Figure 3.

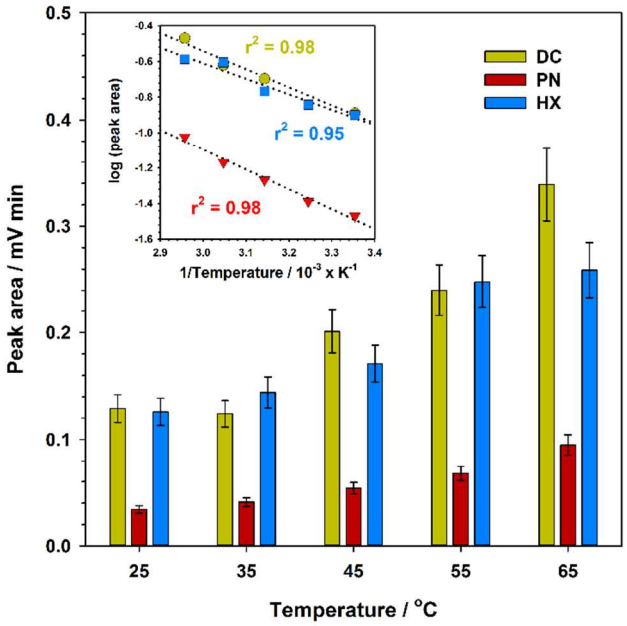

Figure 3 – DC, PN and HX peak variation with the extraction temperature, five different values of temperature were tested: 25, 35, 45, 55 and 65 °C, extractions were performed on 5 g of a bread sample with an aliquot of 100 µL of a solution with 10 mg L^{-1} of three compounds; Inlay – Correlation between the logarithm of peak area (peak is proportional to concentration and thus diffusion) and the inverse of the absolute temperature, for the three vicinal diketones.

According with the obtained results the chosen extraction temperature for the following experiments was 65 °C. Nevertheless, it should be noted that it is also feasible to perform the extraction at lower temperatures, like room temperature, a suitable analytical response is still attained.

Time of extraction

The duration of the extraction process is obviously an important analytical parameter with direct relevance of the methodology's performance. As shown in Figure 4, and studied in previous works,[12] there is an hyperbolic behaviour, typical of a saturation process, i.e. initially there is a linear instrumental response with an increase in the time of extraction and for longer times the response tends to stabilize. For the following extractions 15 minutes were selected as extraction time, since it is a good compromise between sensitivity together with a small time required to perform the analysis. It is important to note that for analytical purposes it is not required to wait for saturation as long as the same extraction time is used.

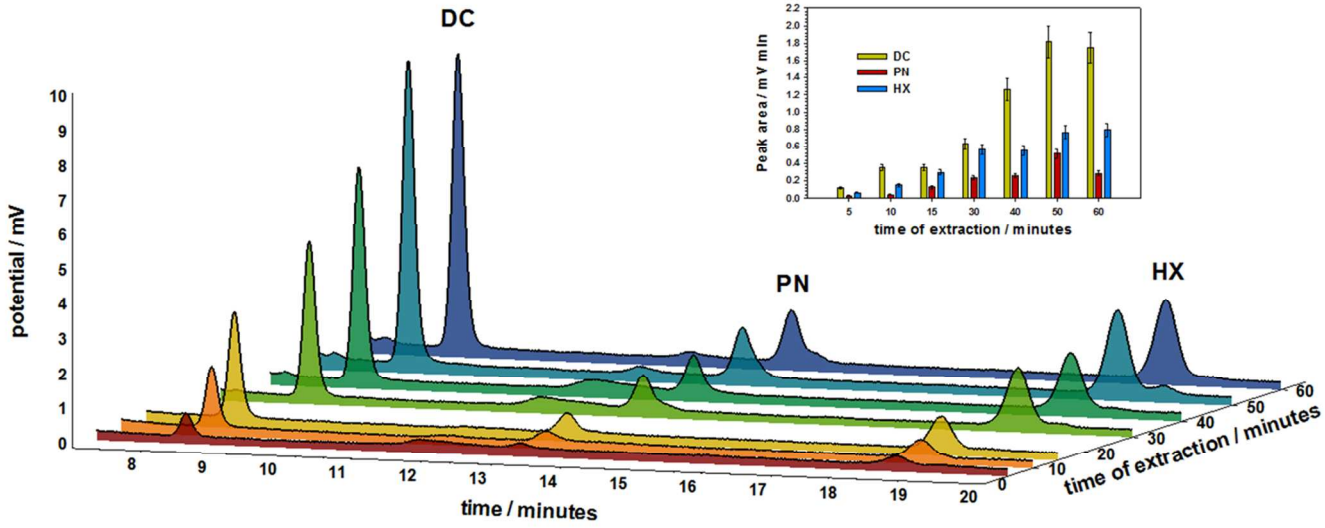

Figure 4 – Chromatograms of several extractions with different extractions times, tested times ranged from 5 minutes up to 1 hour, extractions were performed on 5 g of a bread sample with an aliquot of 100 µL of a solution with 15 mg L^{-1} of DC, PN and HX. Inlay chromatographic peak area variation with time.

Sample size

Different amounts of sample were tested (1, 2, 3 and 5 g) in the GDME chamber. As shown in Figure 5 the obtained results are not changed by the size of the sample that is analysed.

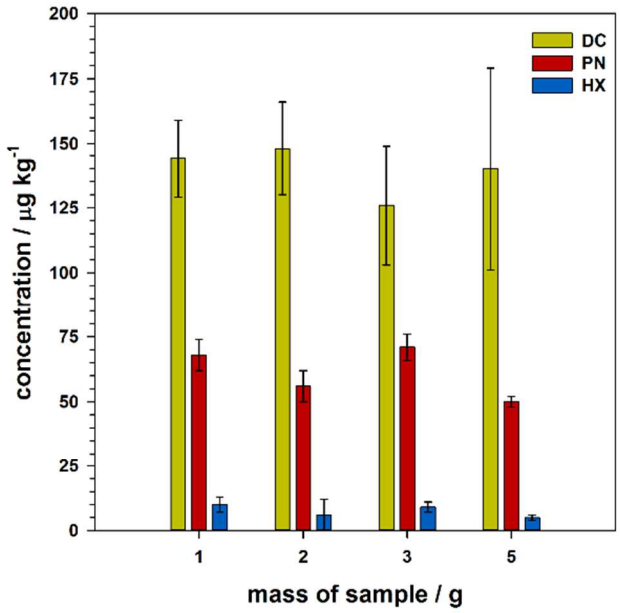

Figure 5 – Determination of DC, PN and HX in a sample of bread using different quantities of sample. For its determination all the standard addition curves had an $r^2 \geq 0.9$.

Figures of merit

The performance of the proposed methodology was estimated by means of matrix-matched calibration curves: extractions were performed over some sample crops of an aged bread (which is as similar as possible with the aimed samples but with minimal quantities of vicinal diketones), placed inside the extraction chamber, with 40 µL of the suitable solution of DC, PN and HX. The figures of merit in terms of linearity ($n \geq 5$), limit of detection (LOD) and limit of quantification (LOQ), and coefficient of variation (CV) are summarized in Table 1. By adjusting the experimental data using linear regression, the obtained values of r^2 were all above 0.990, linear ranges up to 30 µg kg^{-1}. LOD and LOQ were calculated as three and ten times the standard deviation of the intercept by the slope, respectively: a standard sample mass of 5 g was used in the calculus. The CV was evaluated by analysing five replicates of a spiked sample ($n = 5$) on the same day.

Although many methodologies for the determination of α-dicarbonyl in several matrices like beer or wine can be found in literature,[2, 6, 12, 27, 31] with limits of detection mostly in the order of magnitude of the nmol/L or µg/L though lower values can be found in literature,[27, 32] to the best of our knowledge none has been published specifically for the quantitative analysis of bread.

Table 1 – Figures of merit for DC, PN and HX.

Vicinal diketone	r^2	LOD / µg kg^{-1}	LOQ / µg kg^{-1}	CV / %
DC	0.996	6.0	20	4.6
PN	0.991	8.6	29	6.4
HX	0.992	12	38	4.9

Applicability to real samples

The developed methodology was applied to several different bread samples showing its applicability (Table 2). No pretreatment was applied; the standard additions method was used for the quantification of α-dicarbonyl compounds in bread

samples, i.e. by adding small volumes of standard solutions directly to the solid samples.

There are not many studies concerning the levels of these three compounds in bread. Nevertheless the obtained values in Table 2 are similar with the ones that can be found in the scarce existing literature.[33-36]

Table 2 – Concentrations of DC, PN and HX in five different bread samples.

Sample	[DC] / μg Kg^{-1}	[PN] / μg Kg^{-1}	[HX] / μg Kg^{-1}
Bread with dry fruits	99 ± 12	88 ± 29	83 ± 21
Wheat bread	190 ± 17	103 ± 19	< LOQ
Wheat bread with seeds	169 ± 22	165 ± 6	< LOQ
Pão Tigre (*Tijgerbrood*, corn bread)	218 ± 30	118 ± 14	70 ± 22
Broa de Avintes (corn and rye leavened bread)	66 ± 16	90 ± 15	< LOQ

α-diketones evolution with time

Although there is not much information in literature about the evolution of vicinal diketones in bread with time, authors were expecting that with age-related degradation the level of these compounds in bread would wear off. Thus, the content of DC and HX was daily measured in pieces of the same bread sample (Figure 6).

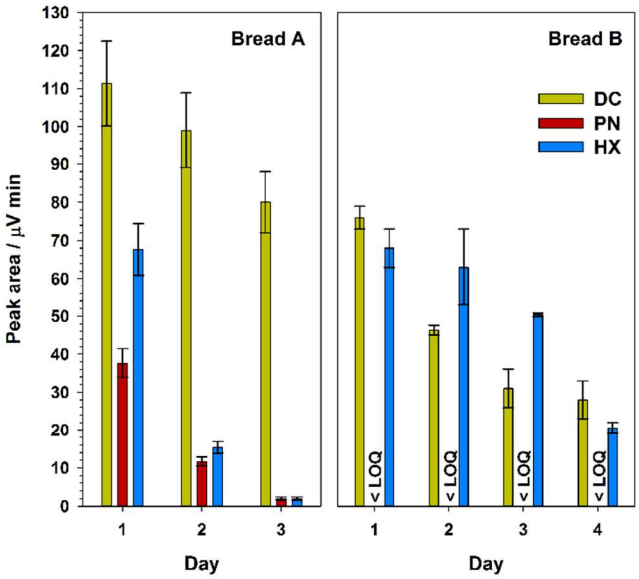

Figure 6 – DC and HX evolution in bread with time. Bread A was a wheat bread with seeds and bread B was a white bread.

Aponte et al.[33] studied the presence of several compounds in chestnut-flour-based sourdoughs after 24 h and 288 h of fermentation. In that study it is also clear the decrease of DC with time, although since only two different times were tested the rate at which it decreases could not be visualized.

Conclusions

GDME, an extraction technique, preferably aimed to volatile and semi-volatile compounds, showed its usefulness in the analysis of solid samples, namely in the determination of α-diketones in bread, an analysis of great importance to which there are not many examples in literature. The proposed methodology was user-friendly, quick and low LODs and LOQs (in the order of magnitude of μg kg^{-1}) were obtained. This specific methodology can be used with different samples other than bread and, furthermore, by changing the chemistry of the acceptor extraction solution and possibly modifying the instrumental technique, GDME can be easily expanded to other solids samples and analytes. Extraction can be further tuned by changing the extraction parameters (temperature, time, membrane material, etc.) to each application.

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