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MONITORING DEGRADATION PROCESSES OF L-ASCORBIC ACID IN ORANGE FRUITS AND JUICES BY CAPILLARY ZONE ELECTROPHORESIS WITH A COMPUTERIZED PNEUMATIC SAMPLING DEVICE

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Abstract. A computerized pneumatic sampler connected to capillary electrophoresis (CE) was used for monitoring the degradation process of L-ascorbic acid (L-AA). The sampler allowed performing multiple injections during one electrophoretic run without high voltage interruption. The decrease in the concentration of L-AA in the samples (orange juice and orange fruits of which the squash was prepared by squeezing the fresh fruit) could be monitored between the runs as well as within the test cycles consisting of 10 injections with 10 min injection intervals. A significant decrease in the L-AA concentration after 72 h storage in a dark and dry room at 17 °C was observed in orange juice, but not in orange squash. The relative standard deviation of the migration time was less than 1% and the intraday and interday r.s.d. of peak areas were respectively 2.1 and 2.8%. The limit of detection was 1 ppm. Automated sampling for the CE method was found to be highly suitable for the determination of L-AA in fruits and fruit products as well as for the monitoring of L-AA degradation process.

Key words: L-ascorbic acid, capillary zone electrophoresis, pneumatic autosampler.

INTRODUCTION

The determination of L-ascorbic acid (L-AA) in fruits and fruit products is very important as L-AA is a vital nutrient. As it is known as an extremely labile substance, it is also vital to monitor L-AA's stability during processing.

The analytical methods for determining L-AA include chemical methods such as monitoring the visual end-point of the 2,6-dichlorophenol–indophenol titration, which, however, does not always prove to be satisfactory enough,

particularly in coloured solutions and if some other reducing impurities are present [1]. Another method, the reaction with 2,4-DNP is a very complicated 6-h process at 20 °C. Temperature and time affect the extent of the reaction but they have a much more profound side effect on the reaction of possible interfering substances. High performance liquid chromatography (HPLC) is the most popular alternative to the standard methods. Currently the most common analytical methods are HPLC separation based on ion-exchange or reversed-phase columns with UV-absorbance, fluorescence or electrochemical detection, and gas chromatography with flame ionization detection [2]. New HPLC methods continue to be developed for the analysis of total vitamin C and its individual vitamers [2]. HPLC is easy and reliable in comparison with the chemical methods. However, it has several drawbacks including the time-consuming conditioning of the columns, the large consumption of solvents, and the high price of columns. As an alternative method, in the current work L-AA's monitoring with capillary zone electrophoresis (CZE) was performed.

Nowadays capillary electrophoresis (CE) is successfully used in various application fields such as biochemistry, biotechnology, pharmaceuticals, and clinical chemistry [3–5]. A number of reports have appeared concerning the application of CE techniques to the examination of food systems. However, judged by lists of the application of the CE presented in recent monographs [6–8] the impact of CE in food science and particularly in the quality control of food and food additives has been minor. Fruits and fruit juices appear to be simple enough matrixes so that their vitamin C content can be reliably determined without performing complicated sample preparation procedures for separating the vitamin from interfering artifacts. This may be partly due to processing and fortification, as a result of which the vitamin C occurs in juice in free (unbound) form, in relatively large concentrations, and potentially as a single vitamer. Recently the determination of total L-AA in foods by CZE with detection limits of 0.25 ppm [2] and 0.5 ppm [9] was reported. This is an exceptionally good result. Detection limits reported by other investigators (e.g. 10 ppm [1, 10] or 1 ppm [11]) are frequently not sufficient when dealing with real life samples.

An important problem in the analysis of L-AA is the instability of vitamin C. The decision to assay a single vitamer by any technique should be weighed carefully according to many investigators [2, 8, 10, 12], who show that the vitamin C content in orange juice has been frequently underestimated when the dehydroascorbic acid (DHAA) content has been ignored. It is a well-known fact that L-AA oxidizes to DHAA. Therefore several antioxidants have been added to the sample before analysis. Although the role of the stabilizers in the determination of L-AA is known to be essential, there are no reports on the consequences if no stabilizing agent was used, i.e. there are no reports on monitoring the degradation process itself.

In this study the degradation process of L-AA occurring in the pure vitamin C sample and the effect of the antioxidant added to the sample were followed by monitoring the sample behaviour during several hours and days. For this purpose we used a computerized pneumatic sampler developed in our laboratory [13] taking advantage of the capability of the sampler to perform multiple injections from the same sample vessel during a single analysis cycle. With the help of the above-mentioned sampler, it was also confirmed that the reactions taking place inside the capillary during the electrophoresis process throughout one testing cycle of multiple injections could be eliminated by adding a stabilizing agent to the running buffer.

Another aim of this work was to reduce detection limits by using a pneumatic sampler taking advantage of its capability to perform sophisticated sampling sequences under computer control. The sampler was used to perform field amplified sample stacking. This enabled us to reduce the detection limit of L-AA to 1 ppm.

EXPERIMENTAL

Chemicals and sample preparation

L-AA was obtained from Sigma Chemical Company, phosphoric acid from YA-Kemia OY, and sodium hydroxide from Chemapol. As a stabilizing agent for the L-AA the L-cysteine [14] from Merck was used. The standard L-AA was dissolved in MilliQ water or buffer to give concentrations in the range 1–350 ppm.

Fruits and juices (Largo from Marli, Finland) were purchased from the local supermarket. In determining the L-AA's content in fruits and juices the products' solutions were filtered through 0.45 μm Millipore filters. The L-AA sample was analysed after the extraction of the sample with MilliQ water followed by filtering through a 0.45 μm filter and adding L-cysteine for stabilization. After that the sample was injected immediately.

Apparatus and method

The CE system consisted of a home-made pneumatic autosampler and a home-made high voltage supply delivering 18 kV. The CE separation was achieved with an untreated fused-silica capillary with 80 cm full length (50 cm to the detector), 50 μm i.d., and 365 μm o.d. (Polymicro Technologies, Phoenix, AR, USA), and an "Isco CV^4" UV detector at 245 nm. The detector signal was digitized and transferred to a "486" type computer via a Keithley "ADC-16" analog-to-digital board. The same board delivered digital signals to the solenoid valves controlling the autosampler. Experiment control and data acquisition were performed with home-made software written in C.

Prior to use, each capillary was rinsed with 1 M NaOH for about 15–20 min, then with MilliQ water, 0.1 M NaOH, and finally with the separation buffer. Between the analyses, capillaries were rinsed with 0.1 M NaOH and subsequently with the separation buffer.

Pneumatic autosampler

The only difference of our system from a classical CE instrument is the sampling device, which enables combining the electrokinetic and hydrodynamic sample introduction methods as well as multiple sampling under computer control according to the sequence generated by computer. Since the sampler design has been described in several papers [13, 15, 16], only a brief outline will be given here. The schematics of the autosampler is shown in Fig. 1.

The sampling is based on the idea of rapid (during 50 ms) replacement of the buffer and sample in the capillary input channel with dimensions of 1 mm i.d. and 1 cm length. The sample introduction to the separation capillary takes place by electroosmosis during the time when the sample is standing still ("hold" time) in the inlet channel of the sampler. As the rinse of the sample past the capillary end takes place under pressure, a part of the sample is introduced into the capillary also hydrodynamically during the rinse time when it passes the capillary end inserted into the inlet channel [15, 16]. The sampler operates on varying sample rinse/hold time ratio by which the capillary inlet is filled with different amounts of the sample. This property of the sampler enables performing head column stacking in addition to common hydrodynamic and electrokinetic sampling.

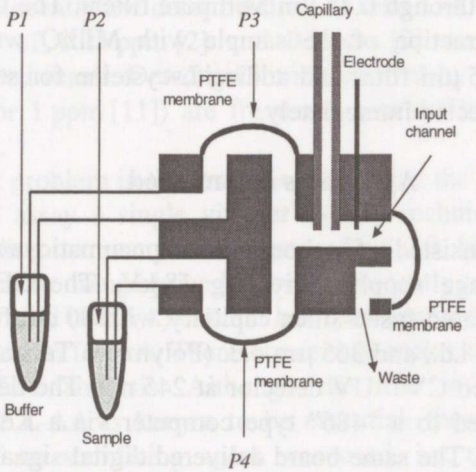

Fig. 1. Schematics of the pneumatic autosampler.

RESULTS

Buffer composition, reproducibility of experiments, and L-AA detection limits

As pK_1 for L-AA is 4.1 and pK_2 is 11.8, it is obvious that the buffer pH must be above 4.2 to ionize the L-AA for performing the CZE separation. Increasing pH value should result in shorter migration times with higher speed of separation. On the other hand, in neutral or alkaline conditions the degradation of L-AA is promoted. Therefore the optimum pH of the running buffer was found to be between 7.0 and 7.5.

The reproducibility of migration times and peak areas in CZE was evaluated by comparing the intraday and interday electropherograms as all the experiments were carried out in optimum conditions determined from the above investigations. The mean value of the migration time of the L-AA peaks was about 9 min. The variation of the migration time of the L-AA peaks was less than 1%. The reproducibility of the peak areas was about 0.4% within one 10-injection testing cycle, 2.1% intraday and 2.8% interdays. Compared with the recent works of Fung & Kim [10], where the reproducibility of peak areas was <5%, it can be concluded that our sampler enabled improving the reproducibility within one series of experiments. This could be attributed to the fact that to operate the sampler it is not necessary to interrupt the electrical field for performing multiple injections in series during one experiment.

During our experiments the detection limit for L-AA was 1 ppm. These results show that the CE method can be used for both qualitative and quantitative determination of L-AA even in rather low concentrations meeting favourably the needs of L-AA determination in real samples where so low concentrations are not likely to occur.

Effect of antioxidant

For eliminating the degradation reactions of L-AA inside the capillary during electrophoretic separation a stabilizing agent (L-cysteine) was added to the running buffer. Results are presented in Fig. 2. It follows from the figure that without L-cysteine the sample demonstrated complicated kinetics when staying in the sample vessel as well as during the separation in the capillary (since the figures are different). Because the demonstration of the monitoring capabilities of the sampler was not the aim of this study, speculations about the nature and essence of the processes (which are probably due to the presence of oxygen in the sample and buffer) are beyond the scope of this paper.

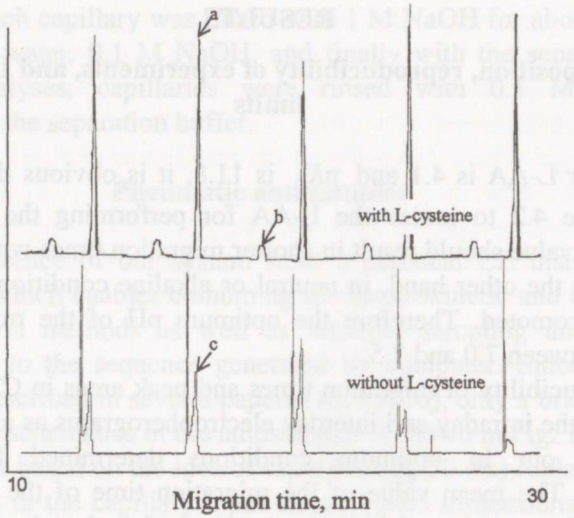

Fig. 2. Monitoring L-AA degradation. Five consecutive injections of L-AA in water solution. Peaks: a, L-AA; b, stabilizer (L-cysteine); c, degradation product(s?). (BGE – 20 mM phosphate buffer, pH = 6.9; 250 mg/L L-cysteine; detector wavelength $\lambda = 245$ nm.)

L-AA in commercial food products made from orange

Results of the monitoring of orange juice and orange squash are presented in Fig. 3, where the confirmation of the L-AA peaks is shown. The peaks of L-AA were identified by characterizing the sample peak in terms of the migration times in comparison with those of the standard, by UV spectrum (L-AA has strong absorption maximum at detection wavelength $\lambda = 245$ nm), and also using the spiking technique as shown in Fig. 3. For the verification of the identification two electropherograms of juice and squash (pure and spiked with L-AA) samples are presented.

Figure 3 indicates that L-AA peak is well separated from the rest of the peaks in the electropherogram meaning that the CZE separation of L-AA in fruits and juices is not remarkably subject to interference by other components in these products. Since multiple injections from the sample were performed subsequently without interrupting the high voltage, the peaks on the pherograms appear as clusters corresponding to the single injections. The dominating feature in the squash pherogram is the L-AA peak, the relatively few interfering peaks are with low intensity, i.e. the chosen UV detector wavelength was favourably discriminating interfering compounds. This is in accordance with findings of other investigators [2]. The concentration of L-AA was 100 mg/L in juice and 150 mg/L in squash.

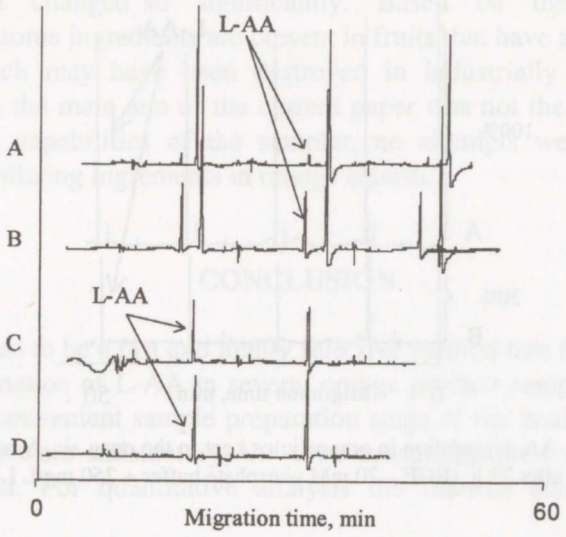

Fig. 3. Electropherograms of orange juice and orange squash. BGE – phosphate buffer with pH = 6.9 + 250 ppm L-cysteine, detector wavelength $\lambda = 245$ nm. A, orange juice filtered and diluted with 50% of MilliQ water (three injections of the same sample); B, the same as A but spiked with 250 ppm L-AA; C, orange squash, filtered and diluted with 50% of MilliQ water (two injections of the same sample); D, the same as C but spiked with 250 ppm L-AA.

Figures 4 and 5 demonstrate how the efficiency of a stabilizer affects the quality of different food products made from orange. In case the orange juice sample has no stabilizer in it and is kept in the open air, it is apparently subject to oxidation. The set of pherograms recorded within one experiment of three

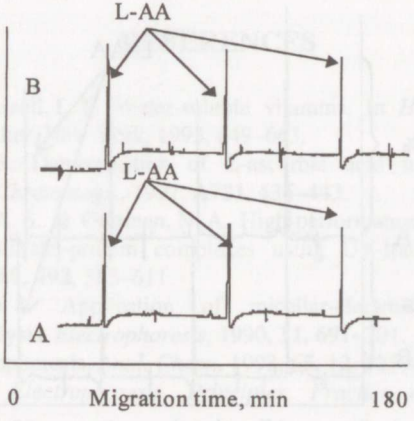

Fig. 4. Kinetics of L-AA degradation within 3 h in orange squash (A) and orange juice (B). (BGE – 20 mM phosphate buffer with 250 mg/L L-cysteine.)

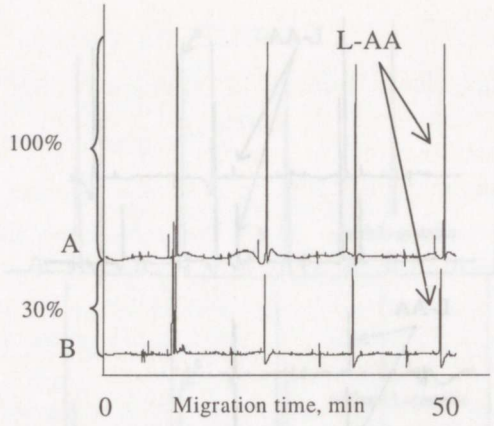

Fig. 5. Kinetics of L-AA degradation in orange juice kept in the open air. A, analysed immediately after preparation; B, after 72 h. (BGE – 20 mM phosphate buffer + 250 mg/L L-cysteine.)

injections (Fig. 4A) demonstrate that significant L-AA degradation processes occur already within a 3-h interval. During this time the intensity of the L-AA peak drops 20%.

On the other hand, adding stabilizer to orange squash seems to be more efficient since the degradation of L-AA was not very extensive within a 3-h interval. The set of pherograms recorded within one experiment of three injections (Fig. 4B) demonstrates that insignificant L-AA degradation processes occur and the intensity of the L-AA peak drops about 5% during this time.

The decrease in the L-AA content was also monitored during a longer period of 3 days. It was found that the L-AA's content in orange juice had decreased about twice compared to fresh juice (Figs. 5 and 6). Its concentration in fruit

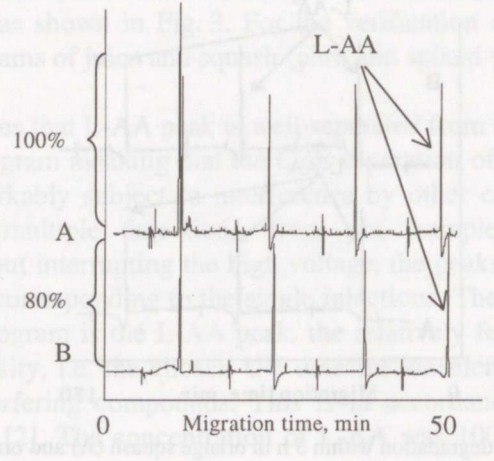

Fig. 6. Kinetics of L-AA degradation in orange squash kept in the open air. A, analysed immediately after preparation; B, after 72 h. (BGE – 20 mM phosphate buffer + 250 mg/L L-cysteine.)

squash had not changed so significantly. Based on these findings we concluded that some ingredients are present in fruits that have a stabilizing effect on L-AA, which may have been destroyed in industrially produced juices. However, since the main aim of the current paper was not the demonstration of the monitoring capabilities of the sampler, no attempts were undertaken to identify the stabilizing ingredients in orange squash.

CONCLUSION

CZE appeared to be a fast and highly selective method free from interferences for the determination of L-AA in several orange product samples. It is possible to avoid the inconvenient sample preparation stage of the analysis. Also, as the detection limits were satisfactory, the time consuming pre-concentration steps can be avoided. For quantitative analysis the internal standard method is preferable.

The design of the pneumatic sampler used in this work enabled easy interfacing of the reactors (for which sample vessels were essential in the current work) to the sample flow path and performing multiple injections under computer generated time sequences. The effect of antioxidants in storing the food products can be easily monitored by this sampler; besides, fresh orange squash appeared to contain natural stabilizing ingredients instrumental in its preservation. Lack of such ingredients in the commercial orange juice was also confirmed by the monitoring.

It can be concluded that CZE, as a method, is relevant for both monitoring the L-AA stability in different solutions and its determination from the real samples. Once again the notorious instability of L-AA was exposed and proven.

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L-ASKORBIINHAPPE LAGUNEMISPROTSESSIDE UURIMINE APELSINIDES JA APELSINIMAHLADES KAPILLAARTSOONELEKTROFOREESIGA KASUTADES ARVUTI POOLT JUHITAVAT PNEUMAATILIST PROOVISISESTUSSEADET

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L-askorbiinhappe (L-AA) lagunemist apelsinides ja apelsinimahlas uuriti kapillaartsoonelektroforeesi abil. Korduvkatsete tegemiseks kasutati arvutiga juhitavat pneumaatilist proovisisestusseadet, et kõrgepingeväli ei katkeks. Võrreldi L-AA sisaldust värsketes produktides (apelsinist pressitud ja tööstuslikult toodetud mahlas) ja 72 tundi säilitatud toodetes (17 °C, pimedas, kuivas ruumis). L-AA sisaldus vähenes nimetatud ajaintervalli jooksul märkimisväärselt tööstuslikus mahlas. Samuti täheldati L-AA sisalduse vähenemist mitmest järjestikusest proovisisestusest koosneva katsetsükli siseselt.

Migratsiooniaegade variatsioon oli vähem kui 1% ja piigipindalade variatsioon päevasiseselt 2,1 ning päevade vaheliselt mõõdetuna 2,8%. Detekteerimispiir oli 1 ppm. Automatiseeritud proovisisestustehnika osutus kapillaarelektroforeesi meetodi korral suurepäraselt sobivaks nii L-AA määramiseks puuviljades ja puuviljaproduktides kui ka L-AA lagunemisprotsesside selgitamiseks.