CHEMIJA. 2016. Vol. 27. No. 1. P. 52–59

Comparison of RPLC and HILIC coupled with tandem mass spectrometry for the determination of ascorbic and dehydroascorbic acids in fruits

Lukas Taujenis,

Vilma Olšauskaitė,

Audrius Padarauskas*

Department of Analytical and Environmental Chemistry, Vilnius University, Naugarduko St. 24, LT-03225 Vilnius, Lithuania

In the present study the potential of reversed phase liquid chromatography (RPLC) and hydrophilic interaction chromatography (HILIC) methods combined with tandem mass spectrometry for the determination of L-ascorbic (L-AA) and dehydroascorbic (DHA) acids in fruit samples was investigated and compared. While both separation modes provide good retention and resolution of acids, the HILIC method is considerably stronger affected by a sample matrix. For the RPLC technique no significant matrix effect was observed in any of the 10-fold diluted extracts from fruit matrices tested. In order to avoid matrix effects in HILIC, the extracts required dilution factors of 50–100. Both methods showed acceptable accuracy for the determination of L-AA in fruits. The obtained recoveries of L-AA ranged from 86 to 105% and from 83 to 97% with RPLC and HILIC, respectively. The RPLC system provided satisfactory recoveries (84–108%) of DHA with good precision (RSD ≤ 4.7%), while in HILIC some problems emerged. HILIC was not sensitive enough to quantify DHA in apple and orange samples. In addition, DHA recoveries ranging from 114 to 135% and unacceptable precision with the RSD up to 24% were obtained for all samples with HILIC.

Keywords: L-ascorbic acid, dehydroascorbic acid, RPLC, HILIC, fruits

INTRODUCTION

Vitamin C is an essential micronutrient which plays a significant role in human metabolic processes. It is mainly involved in several biochemical processes, such as collagen synthesis, iron intake and pulmonary function [1]. It is also recognized as an antioxidant by reducing oxidative free radicals both *in vivo* and *in vitro* [2]. Vitamin C deficiency can cause serious indisposition, that is why the recommended daily dose is 90–100 mg per day [3]. Fresh fruits and vegetables are the most important sources of vitamin C. The main form of vitamin C is L-ascorbic acid (L-AA). L-AA is reversibly oxidized to dehydroascorbic acid (DHA), which exhibits the same biological activity as its reduced form (Fig. 1). Therefore, the concentration of vitamin C is measured as a sum of the amounts of L-AA and DHA [4, 5].

RPLC equipped with UV detection is by far the most commonly employed technique for the determination of L-AA and DHA in various matrices [5–11]. However, the direct determination of both species in a single chromatographic run is difficult because, in contrast to L-AA, DHA exhibits poor UV-absorption properties. The most commonly used approach to overcome that is the so-called subtraction technique, in which

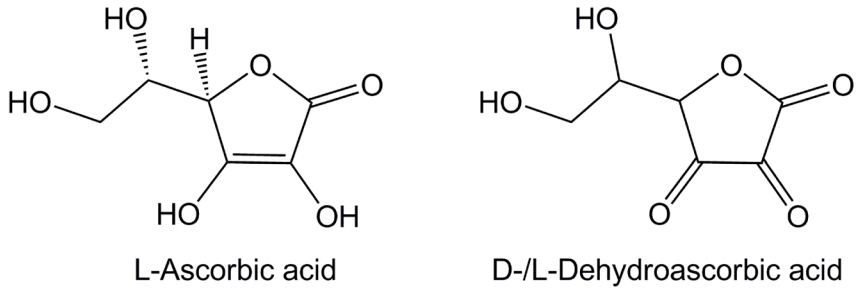

Fig. 1. Structures of acids studied

* Corresponding author. E-mail: audrius.padarauskas@chf.vu.lt

DHA is determined as the difference between the total L-AA after DHA reduction and the L-AA content of the original sample. Several reducing agents, such as dithiothreitol (DTT) [6, 8, 9, 11] or tris(2-carboxy ethyl) phosphine hydrochloride [7, 10], have been employed for this purpose. Alternatively, L-AA and DHA can be determined in a single chromatographic run after pre- or post-column derivatization and subsequent detection by UV [12], electrogenerated luminescence [13], fluorescence [14] or by dual UV-fluorescence [15] detection techniques. However, the requirement for two separate runs and/or the additional reduction/derivatization procedures makes these assays time-consuming and may cause unexpected degradation of the analytes.

The above mentioned problems can usually be avoided by using liquid chromatography coupled to mass spectrometry (LC-MS). The LC-MS technique not only enables the direct detection of both analytes, but also has significant advantages of higher sensitivity and selectivity. During the last decade, LC-MS has become a major technique in analytical laboratories, especially in the biotechnology, pharmaceutical and food industries [16]. It is somewhat surprising that only one work has been published on the use of LC-MS technique for the simultaneous determination of L-AA and DHA in fruits and vegetables [17]. The most recent study by Szultka et al. [18] describes the application of LC-MS to evaluate the stability of L-AA and to characterize its degradation products. However, the proposed method was validated only for L-AA without its application to real samples.

The other drawback of RPLC is that conventional RP stationary phases do not provide sufficient retention of small very polar molecules. Highly aqueous mobile phases [6–11] or ion-pairing additives [12, 19] are therefore required for sufficient retention and adequate resolution. However, such approaches are associated with stationary phase de-wetting under highly aqueous conditions and poorer ionization when coupled to MS.

Hydrophilic interaction chromatography (HILIC) is considered to be an attractive and advantageous chromatographic technique for the separation of very polar and ionizable compounds [20, 21]. This technique uses a polar stationary phase in conjunction with a mobile phase consisting of a polar organic solvent (typically acetonitrile) containing an appreciable amount of water and retains analytes with increasing the order of hydrophilicity. The large percentage of acetonitrile (≥60%) in the HILIC mobile phase enables facilitated solvent evaporation in LC-MS sources and thus often an increase in the analyte response when compared to more aqueous based systems [22]. Taking all these factors into account, the HILIC technique seems to be very promising for the determination of L-AA and DHA. However, despite of the gained popularity of HILIC over the past decade, only a limited number of reports have so far been published on the present topic [23–26]. Moreover, most of them have been focused on the determination of L-AA without taking into account DHA. Only one study dealing with the simultaneous determination of L-AA and DHA in pharmaceutical preparations by HILIC with charged aerosol detection has been published to date [26].

In the present study, RPLC and HILIC techniques combined with tandem mass spectrometry were evaluated and directly compared for the separation and quantification of L-AA and DHA in fruits. In both techniques columns packed with sub-2 μm particles (ultra-high-performance liquid chromatography) were employed, since this modern technology provides faster separations, better resolution, and lower solvent consumption than conventional HPLC.

EXPERIMENTAL

Chemicals and reagents

Ultra-pure water was obtained from a Mili-Q Water Purification System from Millipore (Bedford, MA, USA). Acetonitrile (ACN), formic acid, acetic acid and ammonium acetate were of LC-MS grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethylendiaminetetraacetic acid disodium salt dihydrate (99–101%), DL-dithiothreitol (≥99%), L-Ascorbic acid (≥99%) and dehydroascorbic acid were also from Sigma-Aldrich. Nylon filters of 0.20 μm pore size were purchased from Carl Roth GmbH (Karlsruhe, Germany).

All solutions were prepared in amber glass bottles and vials. Individual stock solutions of L-AA and DHA at 1 mg/mL were freshly prepared in water containing 0.05% (w/v) EDTA (pH 4.5). Working standard solutions were prepared no more than 1 h prior to use by diluting stock solutions in aqueous (for RPLC) or in 80:20 ACN/H_2O (for HILIC) solution containing 0.05% (w/v) EDTA.

Instrumentation

All separations were carried out on a 1290 Infinity UHPLC system connected to a 6410 triple quadrupole mass spectrometer, equipped with an ESI source (Agilent Technologies, USA). The Acquity UPLC HSS T3 (2.1 × 100 mm, 1.8 μm) column (Waters, Milford USA) was employed for the separations in the RPLC mode. An aqueous 5 mmol/L formic acid mobile phase was used at a flow rate of 0.25 mL/min.

The HILIC separation was performed on the Acquity UPLC BEH Amide (2.1 × 100 mm, 1.7 μm) column (Waters). The mobile phase was a mixture of ACN and water (95:5, v/v) containing 5 mmol/L formic acid and set at a flow rate of 0.5 mL/min. For both separation modes the column temperature was 20 °C and the injection volume was 2 μL.

The ESI source operated in the negative ion mode and the selected reaction monitoring (SRM) was performed. The ionization source parameters were optimized with both analytes for each mobile phase composition and flow rate in order to obtain maximum signal intensity. The nebulizer pressure, capillary voltage and drying gas flow rate were identical for both separation modes (60 psi, 4000 V and 10 L/min, respectively), while the drying gas temperatures in RPLC and HILIC were set at 320 and 280 °C, respectively. Data were acquired and processed using the MassHunter software (Agilent).

Sample preparation

Fruits were purchased from a local supermarket. The samples were prepared according to a slightly modified procedure described by Fenoll et al. [17]. Briefly, the representative amount of the sample (~100 g) was homogenized using a blender and 1.00 g of fine homogenate was mixed with 10 mL of 0.05% (w/v) EDTA solution in a centrifugation tube. The mixture was thoroughly vortexed for 1 min and then centrifuged at 10000 × g for 10 min, using a centrifuge Sigma 3K30 (Sigma Laborzentrifugen GmbH, Germany). The supernatant was filtered through a 0.2 μm nylon syringe filter and diluted at the appropriate ratio with an aqueous (for RPLC) or with 80:20 ACN/H_2O (for HILIC) solution containing 0.05% (w/v) EDTA. The samples were kept in amber vials and were analyzed immediately after extraction.

RESULTS AND DISCUSSION

Characterization of DHA

Commercial L-AA is a pure compound and can be easily stabilized in an aqueous solution under appropriate conditions, while in the commercial DHA a certain amount of dimer (2DHA) with two bicyclic monomers connected through two oxygen atoms may be present [4]. Several previous studies have shown that 2DHA is readily hydrolysed to DHA in slightly acidic or neutral aqueous solutions [27, 28]. Thus, a freshly prepared DHA solution may contain at least two compounds, which amounts could change in time.

In order to evaluate the species formed from DHA in the solution as well as their stability and possible transformations, DHA was dissolved in aqueous 10 mmol/L formic acid (pH 2.9), 10 mmol/L ammonium acetate buffer (pH 5.0) and 10 mmol/L ammonium acetate (pH 7.0), aged for an appropriate time, then diluted with 80:20 (v/v) ACN:H_2O and analyzed by HILIC-ESI-MS. For the freshly prepared from the solid DHA solution at pH 2.9 two peaks eluting at 0.98 and 1.90 min were observed (Fig. 2a). After about 30 min incubation, the first eluting DHA species was completely transformed into later eluting compound and no further changes in the chromatographic profile were observed after an aging period at least up to 3 h. By contrast, the chromatographic profiles obtained after dissolution of DHA at higher pHs showed only one peak eluting at 1.90 min with no further changes in time (Fig. 2b).

The negative ion ESI mass spectra corresponding to the peaks in Fig. 2a are presented in Fig. 3. The full scan spectrum of peak 1 (Fig. 3a) displays a predominant ion at *m/z* 347.0 which corresponds to the molecular ion of DHA dimer $[2DHA-H]^-$. The less abundant ion at *m/z* 383.1 corresponds to the water adduct $[2DHA+2H_2O-H]^-$ of DHA dimer, while ions at *m/z* 521.1 and 695.1 may be attributed to the trimer $[3DHA-H]^-$ and tetramer $[4DHA-H]^-$ species, respectively. Furthermore, a deprotonated monomer ion $[DHA-H]^-$ is also observed at *m/z* 173.0. It is likely that trimer and tetramer clusters are formed by non-covalent aggregation through multiple hydrogen bonds before or after the spray process. The full scan

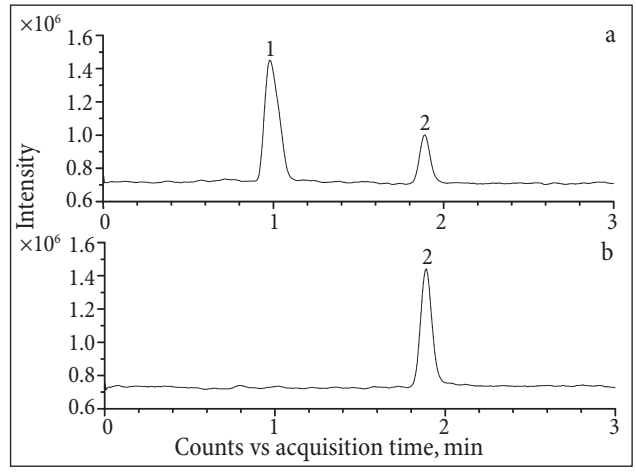

Fig. 2. Total ion chromatograms (full scan mode) of the DHA standard (100 mg/L) obtained in the HILIC mode. (a) DHA dissolved in aqueous 10 mmol/L formic acid (pH 2.9); (b) DHA dissolved in aqueous 10 mmol/L ammonium acetate buffer (pH 5.0). For chromatographic conditions see the Instrumentation section

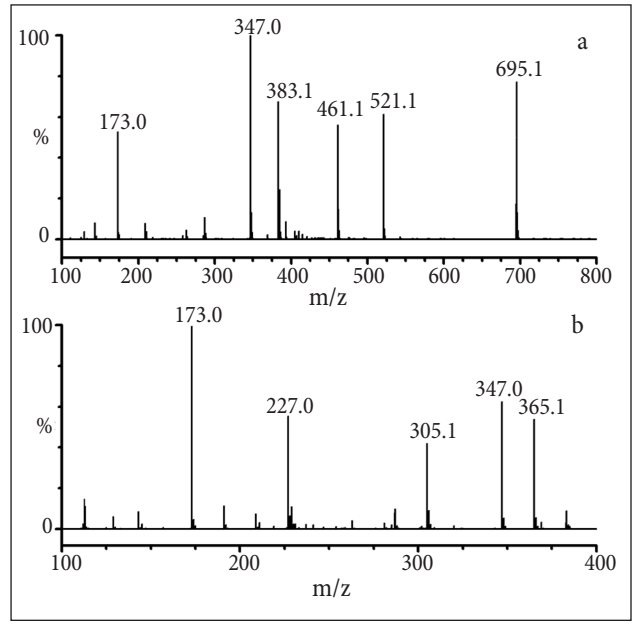

Fig. 3. Negative ESI mass spectra corresponding to peak 1 (a) and peak 2 (b) of Fig. 2a

mass spectrum of peak 2 (Fig. 3b) depicts the most abundant molecular ion $[DHA-H]^-$ (*m/z* 173.0) and three less abundant ones at *m/z* 227.0, 347.0 and 365.1 whose structures correspond to $[DHA+3H_2O-H]^-$, $[2DHA-H]^-$ and $[2DHA+H_2O-H]^-$, respectively. The obtained results suggest that peaks 1 and 2 might be attributed to the dimeric and monomeric forms of DHA, respectively.

Similar experiments were also performed in the RPLC mode. The only difference was that in RPLC aqueous solutions of DHA were injected. For the freshly prepared DHA solution at pH 2.9 two peaks were observed, while the chromatographic profiles obtained for DHA standards dissolved

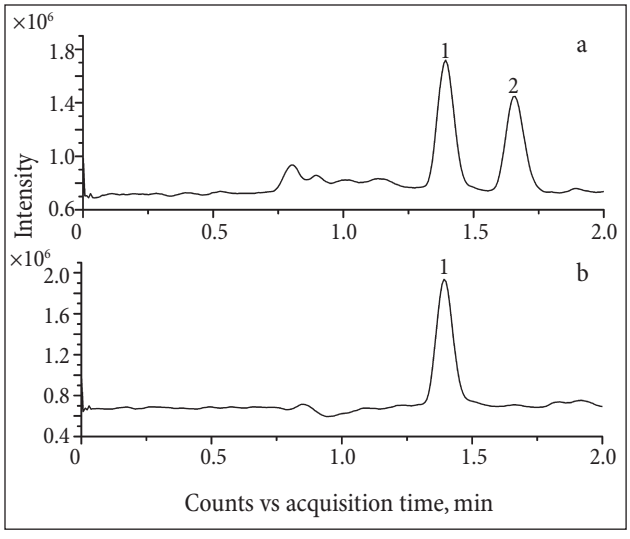

Fig. 4. Total ion chromatograms (full scan mode) of the DHA standard (100 mg/L) obtained in the RPLC mode. (a) DHA dissolved in aqueous 10 mmol/L formic acid (pH 2.9); (b) DHA dissolved in aqueous 10 mmol/L ammonium acetate buffer (pH 5.0). For chromatographic conditions see the Instrumentation section

at higher pHs showed only the first eluted peak (Fig. 4).

The full scan mass spectrum of peak 1 of Fig. 4a depicts the most abundant molecular ion $[DHA\text{-}H]^-$ at *m/z* 173.0 and a less abundant ion $[DHA+3H_2O\text{-}H]^-$ at *m/z* 226.9 (Fig. 5a). The mass spectrum of peak 2 of Fig. 4a displays a predominant ion at *m/z* 364.9 which corresponds to the water adduct $[2DHA+H_2O\text{-}H]^-$ of DHA dimer (Fig. 5b). The less abundant ion at *m/z* 191.0 corresponds to the water adduct $[DHA+H_2O\text{-}H]^-$. No cluster formation was observed in RPLC likely because the hydrogen bonding between DHA molecules is suppressed in the aqueous phase.

Based on the above results, it can be concluded that the formation of DHA dimer can be avoided by aging the freshly prepared solution of DHA for about 30 min before further use or by dissolving DHA at pHs higher than 4.

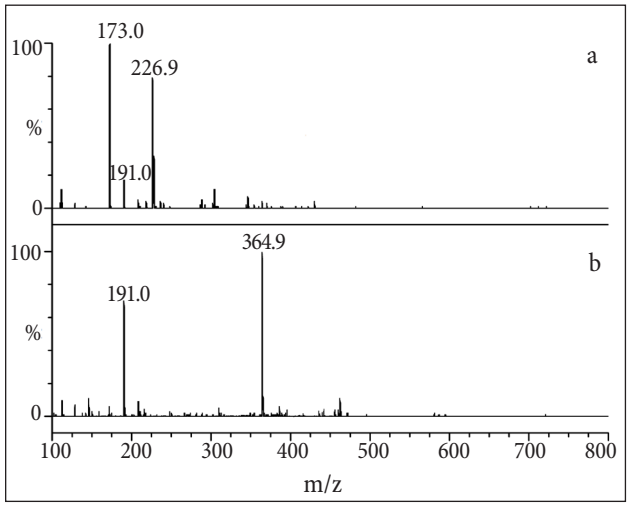

Fig. 5. Negative ESI mass spectra corresponding to peak 1 (a) and peak 2 (b) of Fig. 4a

Finally, it is reasonable to raise the question whether the commercial DHA can be used as a standard for quantification of DHA. In order to address to this question, the DHA standard at three concentration levels (1.0, 2.5 and 10 mg/L) was reduced to L-AA with DTT in ammonium acetate buffer (pH 5) as described in [29] and yields of L-AA were measured. The obtained recoveries of L-AA ranged from 97 to 99% indicating the suitability of the commercial DHA for quantification purposes.

LC-MS

Series of experiments were performed to optimize the separation and signal response conditions for L-AA and DHA acids in both, RP and HILIC separation modes, using isocratic elution. For RPLC separations the Acquity UPLC HSS T3 column was selected. The universal, silica-based C18 bonded phase used for the Acquity HSS T3 sorbents is compatible with the aqueous mobile phase and exhibits superior retentivity of very polar compounds. As expected, both acids were most strongly retained under anion suppression conditions using the acidified (5 mmol/L HCOOH) 100% aqueous mobile phase.

For HILIC separations the Acquity UPLC BEH Amide column was employed which, compared to conventional bare silica HILIC phases, provides enhanced retention of acidic solutes.

The final chromatographic conditions of the methods compared here are detailed in the Instrumentation section and the resulting chromatograms are shown in Fig. 6. Considering the twice higher mobile phase flow rate employed in HILIC, this technique provides significantly better retentivity for both analytes. Finally, it is interesting to note that the elution order is not reversed between RPLC and HILIC indicating that for the present analytes the two separation modes are not orthogonal despite the different retention mechanisms. The elution order of the acids obtained in our study by HILIC agrees with those found by Nováková et al. [26]. However, some contradictory results have been reported with respect to the elution order of L-AA and DHA in RPLC employing conventional C18 phases [17, 18]. DHA was eluted before L-AA with a mobile phase of 0.2% formic acid in water [17], whereas an opposite elution order was observed using a mobile phase of 0.085% formic acid in methanol/water (30:70 *v/v*) [18]. The reason for the reversal in the elution order of L-AA and DHA under an identical separation mode is not known. Clearly, some of analyte/mobile phase properties (e. g. pK_a and pH values) are affected by the solvent composition. Thus, it is likely that the change in the mobile phase composition (pure water vs methanol/water) affects the analytes in different extent causing elution order reversal.

The mass spectra obtained for analyte standard solutions showed major deprotonated ions *m/z* = 175 for L-AA and *m/z* = 173 for DHA. Next, the full scan product-ion spectra of the analytes were investigated to determine the most abundant product ions for SRM. Major 115 and 87 product

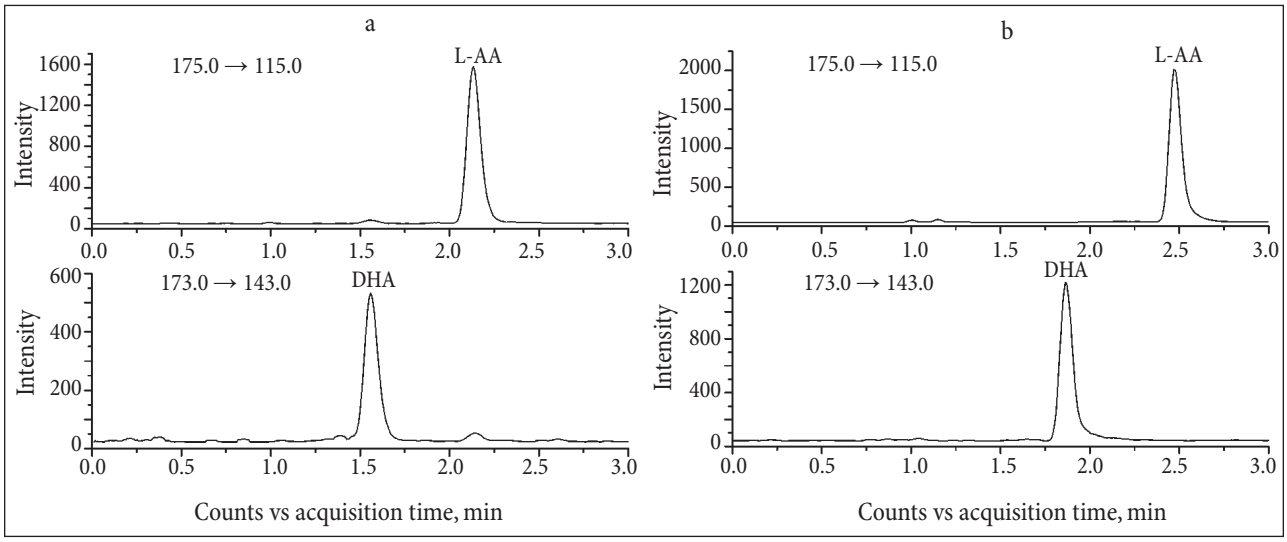

Fig. 6. Extracted ion chromatograms illustrating separation of DHA (1.0 mg/L) and L-AA (2.0 mg/L) by RPLC (a) and HILIC (b). For chromatographic conditions see the Instrumentation section

ions were observed for L-AA, whereas DHA collision induced dissociation led to 143 and 113 fragment ions. Fragmentor voltages and collision energies were optimized for each compound to obtain two SRM transitions. The most intense product ion was used for quantification, whereas the second one was used to complete the identification. The SRM transitions, fragmentor voltages and the collision energies of each analyte are presented in Table 1.

Table 1. **Optimal SRM transition parameters for studied acids (selected quantification transitions are in bold)**

Analyte	Transition, m/z	Fragmentor voltage, V	Collision energy, eV
L-AA	**175 → 115**	**110**	**5**
	175 → 87	110	17
DHA	**173 → 143**	**90**	**4**
	173 → 113	90	3

Stability and matrix effects

Stability is a key problem of L-AA and DHA analysis because the compounds are known to be very unstable in an aqueous solution. L-AA can be stabilized in the solution by adding various stabilizing agents, among which *m*-phosphoric acid (MPA) has been most widely used [5]. However, MPA is not compatible with the HILIC separation mode due to its limited solubility in acetonitrile-rich mobile phases. Although MPA concentrations in the range of 5–10% are usually used for stabilization, even at 1% concentration it precipitated in our HILIC mobile phase. A similar behaviour was also observed in other study [24]. Other stabilizers, such as *o*-phosphoric acid [24], EDTA [17] or trichloroacetic acid [30], were also found to be efficient. In this study, EDTA was chosen as a stabilizer because of its good compatibility with both RP and HILIC mobile phases as well as with MS. The stability of L-AA and DHA in the aqueous 0.05% (w/v) EDTA solution (pH 4.5) was briefly investigated. For the three concentration levels tested (1.0, 2.5 and 10 mg/L) both analytes remained stable for at least two hours.

One significant drawback of ESI-MS is that the ionization process is greatly affected by coeluting matrix compounds [31]. The matrix effect typically results in the suppression or, less frequently, the enhancement of the analyte signal. In this work, the matrix effect was evaluated by the post-extraction spike method [32]. Ten-fold diluted extracts from three fruit matrices (apple, kiwi and orange) were spiked with analytes at different concentrations (2.5–25 mg/L for L-AA and 0.5–5 mg/L for DHA) and the slopes of the calibration plots were compared with those obtained from pure solution standards. Table 2 compares the slope ratios matrix/solvent for both analytes in the matrices evaluated. The results show that for the RPLC technique no significant matrix effect was observed in any of the 10-fold diluted matrices tested. By contrast, using the HILIC mode, significant matrix effects (except for L-AA in kiwi), from 29 to 68% signal suppression, were obtained for both acids in the orange and for DHA in the kiwi matrix. Less common behaviour was observed for the apple extract where the signal response was enhanced by the matrix. The HILIC method is considerably stronger affected by the matrix, probably due to highly polar compounds of the fruit matrices being strongly retained in HILIC and therefore more likely to co-elute with the analytes studied. In addition, under HILIC conditions sequential injection of 10-fold diluted extracts was accompanied by a gradual increase in the system back pressure. After 20 injections the column back pressure has increased approximately by 14% over its initial value. This was accompanied by retention time shifts and broader peaks. The reason for this behaviour can most

Table 2. **Slope ratios between matrix-matched (S_m) and solvent (S_s) calibration (n = 3, extract dilution factor of 10)**

LC method	Matrix	S_m/S_s	
		L-AA	DHA
RP	Apple	0.99	0.96
	Kiwi	0.93	1.05
	Orange	0.96	0.98
HILIC	Apple	1.26	1.50
	Kiwi	1.06	0.71
	Orange	0.66	0.32

likely be attributed to the precipitation of polar matrix compounds in the highly organic HILIC mobile phase. Although the flushing of the column with a 20 minute gradient from 10 to 100% water was sufficient to restore the initial column performance, the need of periodic column flushing makes the method hardly suitable for routine applications. Thus, the standard addition protocol often used for quantification purposes to compensate for matrix effects cannot be used in this case. Common approaches, which may be employed to reduce matrix effects and to improve extract compatibility with an organic-rich mobile phase, include an additional extract clean-up procedure, further diluting of the extract or taking less amount of the sample for the extraction. It should be noted that the second and third approaches can actually be considered as the same. Clearly, an effective sample clean-up procedure is the ideal approach to remove matrix effects and to enhance extract compatibility with the HILIC mobile phase, but extensive sample preparation steps may be time-consuming and bring about the risk of loss of unstable analytes. Furthermore, it is a difficult or perhaps even impossible task to find the clean-up protocol for the effective isolation of the polar analytes from other polar matrix compounds. In a recent study by Stahnke et al. [33] the relationship between matrix concentration and suppression of ESI-MS was investigated for 39 pesticides in several fruit and vegetable matrices. The authors showed that the slope but not the bias of calibration curves is affected by the matrix and the extent of matrix effect does not depend on analyte concentration. Thus, a complete elimination of all matrix effects by appropriate dilution of sample extracts is possible. Although this approach leads to reduced detectability, it was still employed in our study. It was found that to avoid precipitation of matrix compounds and to minimize signal suppression/enhancement effects for both analytes to an acceptable level (≤10%), the extracts of kiwi must be diluted by a factor of 50, whereas the extracts of apple and orange required a dilution factor of 100. Thus, dilution of the extracts by a factor of 100 was used in subsequent HILIC separations.

Analytical performance

Both methods were evaluated for linearity, LODs and LOQs, accuracy and precision. Linearity was measured with the solvent-based seven-point calibration curve (three replicates). LODs and LOQs were evaluated from the calibration curves based on the standard deviation (SD) of the response (y-intercepts of regression lines) and the slope *S* according to the following formulas: LOD = 3.3(SD/*S*) and LOQ = 10(SD/*S*). Linearity ranges as well as LODs and LOQs provided for L-AA by RPLC and HILIC are similar, while for DHA only approximately twice lower LOD and LOQ values were obtained by HILIC (Table 3). Oftentimes, the highly volatile organic mobile phase in HILIC provides higher desolvation efficiency, leading to a significant sensitivity increase in ESI-MS when compared to RPLC [34]. However, expected gain in sensitivity under HILIC was not observed in our case. Due to the complexity of the ESI process, it is difficult to explain such behaviour. Of course, solvent volatility is not the unique factor that determines sensitivity differences between both chromatographic modes. The response should be inversely proportional to analyte pK_a, a measure of analyte acidity [35]. In the presence of a large amount of ACN both acidic and basic analytes should exhibit higher pK_a values compared with the aqueous pK_a. This means that under HILIC basic analytes become more basic exhibiting a higher ionization degree, while acidic ones become less acidic with lower ionization degree. Thus, this factor may affect the response for basic and acidic analytes in opposite directions and the gain in sensitivity between HILIC and RPLC for basic compounds may be much more significant than for acidic ones. To date, most studies dealing with comparison of ESI-MS sensitivity between RPLC and HILIC have focused on basic analytes and, as expected, enhanced sensitivity with positive ion ESI-MS in HILIC has been obtained for most compounds [35–37]. Recently, Huffman et al. [38] extensively examined the effect of polar solvents on a negative ion ESI response of small acidic compounds. It was found that for the majority of test compounds responses followed the order methanol > water > acetonitrile > acetone. The LODs obtained in this work for L-AA and DHA by

Table 3. **Calibration data, LODs and LOQs for the HILIC and RPLC methods (n = 3)**

Parameter	HILIC		RPLC	
	L-AA	DHA	L-AA	DHA
Linear range, µg/mL	0.10–50.0	0.10–25.0	0.10–50.0	0.10–25.0
Regression equation	$y = 2401x - 448$	$y = 754.6x - 48.3$	$y = 2368x - 639$	$y = 597.0x - 23.7$
R^2	0.9988	0.9990	0.9991	0.9943
LOD, ng/mL	34	15	31	26
LOQ, ng/mL	103	44	93	90

RPLC-MS are comparable with those achieved using a similar technique [17]. Unfortunately, there are no reports on HILIC-MS of the acids studied. In conclusion, considering 10-times smaller dilution factors required for RPLC to avoid matrix effects, this technique exhibits overall better sensitivity.

Finally, the analysis of three samples of fruits of different types were analysed for L-AA and DHA by both methods (Table 4). In order to evaluate the accuracy of the methods recovery tests were carried out at three concentration levels. The amounts of L-AA determined in all three samples showed a good correlation between both methods. The obtained recoveries of L-AA ranged from 86 to 105% and from 83 to 97% with RPLC and HILIC, respectively. Both techniques showed adequate precision for L-AA with RSD values ranged from 1.2 to 4.8%. Slightly higher RSDs obtained with HILIC might be attributed to the 10-times lower L-AA concentrations present in the final extracts taken for the analysis. Thus, it can be concluded that both methods have shown acceptable accuracy and precision for the determination of L-AA in fruits but the RPLC should be preferred due to the better sensitivity.

Much more significant differences between the two methods were observed by the DHA analysis. The RPLC system provided satisfactory recoveries (84–108%) of DHA with good precision (RSD ≤ 4.7%), while in HILIC some problems emerged. First of all, the HILIC was not sensitive enough to quantify DHA in 100-fold diluted extracts of apple and orange samples. Furthermore, considerably higher than 100% DHA recoveries ranging from 114 to 135% and unacceptable precision with the RSD up to 24% were obtained for all samples with HILIC. It should be noted that when only L-AA to the samples was added, a slight increase in the initial DHA peak was observed under HILIC conditions. However, there was no correlation between the concentration of L-AA added and the peak area of DHA formed. In addition, when a recovery test by adding of only DHA to the apple sample was performed with HILIC, satisfactory recoveries ranging from 91 to 105% (RSD ≤ 7.9%) were obtained. These results suggest that most likely an unexpected transformation of added L-AA to DHA during the separation in the acetonitrile-rich HILIC mobile phase takes place. To explain the transformation mechanism further more detailed experiments are planned in our lab.

CONCLUSIONS

RPLC and HILIC techniques were evaluated and compared for the LC-MS determination of L-AA and DHA acids in fruit samples. Both methods demonstrated sufficient resolution of the acids but the HILIC technique provided better retentivity. When working with solvent-based standard solutions, both RPLC and HILIC separation modes have shown comparable linearity and sensitivity. One of the major advantages of using RPLC over HILIC with ESI-MS is the higher tolerance to the fruit sample matrix. In HILIC much more significant signal suppression was observed. In addition, due to

Table 4. **Average values of L-AA and DHA determined (mg/100 g in fresh weight basis) in different fruits, accuracy and precision data (n = 3)**

Sample	Analyte	RPLC			HILIC		
		Found, mg/100 g	Added, mg/100 g	Recovery, %	Found, mg/100 g	Added, mg/100 g	Recovery, %
Apple	L-AA	15.5 (2.4)[a]	10.0	86 (3.1)	15.1 (4.5)	10.0	83 (4.8)
			25.0	98 (2.7)		25.0	94 (5.2)
			100	95 (2.5)		100	93 (4.4)
	DHA	2.33 (3.8)	2.50	84 (4.4)	ND[b]	2.50	135 (15.2)
			10.0	92 (3.2)		10.0	122 (18.5)
			25.0	88 (3.5)		25.0	119 (13.6)
Kiwi	L-AA	108 (1.6)	10.0	105 (1.2)	102 (3.5)	10.0	89 (3.6)
			25.0	101 (1.8)		25.0	86 (2.9)
			100	95 (1.5)		100	95 (3.3)
	DHA	23.1 (3.6)	2.50	100 (2.8)	25.5 (8.2)	2.50	129 (22.0)
			10.0	91 (3.4)		10.0	118 (16.4)
			25.0	95 (2.0)		25.0	114 (12.2)
Orange	L-AA	45.4 (2.3)	10.0	94 (3.4)	43.0 (3.8)	10.0	95 (3.5)
			25.0	92 (3.2)		25.0	88 (3.1)
			100	98 (1.9)		100	97 (2.5)
	DHA	3.86 (4.5)	2.50	108 (4.7)	ND	2.50	126 (24.2)
			10.0	104 (4.1)		10.0	120 (14.0)
			25.0	98 (3.8)		25.0	119 (18.4)

[a] Values in parentheses are %RSD (n = 3).

[b] Not determined (below the LOQ).

the precipitation of polar matrix compounds in the highly organic HILIC mobile phase a gradual increase in the system back pressure appeared. Although matrix effects were eliminated by 100-fold dilution of sample extracts, such extra dilution enhances LOQs/LODs for the analytes. Therefore HILIC was not sensitive enough to quantify DHA in apple and orange samples. Finally, overestimation of DHA with poor precision was obtained in the HILIC mode.

Received 6 October 2015
Accepted 22 October 2015

References

1. H. E. Sauberlich, *Annu. Rev. Nutr*., **14**, 371 (1994).
2. T. Byers, G. Perry, *Annu. Rev. Nutr*., **12**, 139 (1992).
3. A. C. Carr, B. Frei, *Am. J. Clin. Nutr.*, **69**, 1086 (1999).
4. J. C. Deutsch, *J. Chromatogr. A*, **881**, 299 (2000).
5. L. Nováková, P. Solich, D. Solichová, *Trends Anal. Chem.*, **27**, 942 (2008).
6. V. Gökmen, N. Kahraman, N. Demir, J. Acar, *J. Chromatogr. A*, **881**, 309 (2000).
7. A. Karlsen, R. Blomhoff, T. E. Gundersen, *J. Chromatogr. B*, **824**, 132 (2005).
8. Y. Hernández, M. Gloria Lobo, M. González, *Food Chem*., **96**, 654 (2006).
9. I. Odriozola-Serrano, T. Hernández-Jover, O. Martín-Belloso, *Food Chem*., **105**, 1151 (2007).
10. K. K. Chebrolu, G. K. Jayaprakasha, K. S. Yoo, J. L. Jifon, B. S. Patil, *LWT-Food Sci. Technol.*, **47**, 443 (2012).
11. V. Spínola, B. Mendes, J. S. Câmara, P. C. Castilho, *Anal. Bioanal. Chem.*, **403**, 1049 (2012).
12. M. G. Gioia, P. Andreatta, S. Boschetti, R. Gatti, *J. Pharm. Biomed. Anal.*, **48**, 331 (2008).
13. M. Zorzi, P. Pastore, F. Magno, *Anal. Chem*., **72**, 4934 (2000).
14. J. Iglesias, M. J. González, I. Medina, *Eur. Food Res. Technol.*, **223**, 781 (2006).
15. S. I. D. Simões, C. V. Eleutério, M. E. M. Cruz, M. L. Corvo, M. B. F. Martins, *Eur. J. Pharmacol. Sci.*, **18**, 185 (2003).
16. A. K. Malik, C. Blasco, Y. Picó, *J. Chromatogr. A*, **1217**, 4018 (2010).
17. J. Fenoll, A. Martínez, P. Hellín, P. Flores, *Food Chem.*, **127**, 340 (2011).
18. M. Szultka, M. Buszewska-Forajta, R. Kaliszan, B. Buszewski, *Electrophoresis*, **35**, 585 (2014).
19. J. Lykkesfeldt, *Anal. Biochem.*, **282**, 89 (2000).
20. P. Hemström, K. Irgum, *J. Sep. Sci.*, **29**, 1784 (2006).
21. B. Dejaegher, Y. V. Heyden, *J. Sep. Sci.*, **33**, 698 (2010).
22. R. Simon, Q. Enjalbert, J. Biarc, J. Lemoine, A. Salvador, *J.Chromatogr. A*, **1264**, 31 (2010).
23. A. Tai, E. Gohda, *J. Chromatogr. B*, **853**, 214 (2007).
24. L. Nováková, D. Solichová, S. Pavlovičová, P. Solich, *J. Sep. Sci.*, **31**, 1634 (2008).
25. A. I. R. N. A. Barros, A. P. Silva, B. Gonçalves, F. M. Nunes, *Anal. Bioanal. Chem.*, **396**, 1863 (2010).
26. L. Nováková, S. Solichová, P. Solich, *J. Chromatogr. A*, **1216**, 4574 (2009).
27. N. Cioffi, I. Losito, R. Terzano, C. G. Zambonin, *Analyst*, **125**, 2244 (2000).
28. L. Wechtersbach, T. Polak, N. Poklar Ulrih, B. Cigić, *Food Chem.*, **129**, 965 (2011).
29. L. Wechtersbach, B. Cigić, *J. Biochem. Biophys. Methods*, **70**, 767 (2007).
30. G. Burini, *J. Chromatogr. A*, **1154**, 97 (2007).
31. P. J. Taylor, *Clin. Biochem*., **38**, 328 (2005).
32. A. Van Eeckhaut, K. Lanckmans, S. Sarre, I. Smolders, Y. Michotte, *J. Chromatogr. B*, **877**, 2198 (2009).
33. H. Stahnke, S. Kittlaus, G. Kempe, L. Alder, *Anal. Chem*., **84**, 1474 (2012).
34. H. P. Nguyen, K. A. Schug, *J. Sep. Sci.*, **31**, 1465 (2008).
35. A. Periat, J. Boccard, J. L. Veuthey, S. Rudaz, D. Guillarme, *J. Chromatogr. A*, **1312**, 49 (2013).
36. C. Apostolou, C. Kousoulos, Y. Dotsikas, Y. L. Loukas, *Biomed. Chromatogr*., **22**, 1393 (2008).
37. N. Gray, J. Heaton, A. Musenga, D. A. Cowan, R. S. Plumb, N. W. Smith, *J. Chromatogr. A*, **1289**, 37 (2013).
38. B. A. Huffman, M. L. Poltash, C. A. Hughey, *Anal. Chem*., **84**, 9942 (2012).

Lukas Taujenis, Vilma Olšauskaitė, Audrius Padarauskas

RPLC-MS IR HILIC-MS METODŲ PALYGINIMAS ASKORBO IR DEHIDROASKORBO RŪGŠTIMS VAISIUOSE NUSTATYTI

S a n t r a u k a

Atvirkščių fazių (RPLC) ir hidrofilinės sąveikos (HILIC) skysčių chromatografijos-tandeminės masių spektrometrijos metodai ištirti ir palyginti L-askorbo (L-AA) bei dehidroaskorbo (DHA) rūgštims vaisiuose nustatyti. Abiem metodais rūgštys sulaikomos ir gerai atskiriamos, tačiau HILIC metodas yra stipriau veikiamas mėginių matricos. Analizuojant 10 kartų praskiestus vaisių ekstraktus RPLC metodu, mėginio matricos įtaka nereikšminga. Matricos poveikiui pašalinti HILIC metodu ekstraktus būtina skiesti 50–100 kartų. Abiem metodais L-AA nustatymo tikslumas yra tinkamas kiekybinei analizei. L-AA išgavos iš vaisių sudaro 86–105 % (RPLC metodas) ir 83–97 % (HILIC metodas). DHA nustatymo RPLC metodu charakteristikos taip pat geros (išgavos 84–108 %, RSD ≤ 4,7 %). Tačiau HILIC metodo jautris nepakankamas DHA obuoliuose ir apelsinuose nustatyti. Be to, DHA nustatymo HILIC metodu tikslumas (išgavos 114–135 %) ir glaudumas (RSD ≤ 24 %) netenkina kiekybinei analizei keliamų reikalavimų.