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Development and validation of a hydrophilic interaction chromatography-mass spectrometry assay for taurine and methionine in matrices rich in carbohydrates☆

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Abstract

A new procedure based on hydrophilic interaction chromatography coupled to tandem mass spectrometry (ionisation process by pneumatically assisted electrospray in negative ion mode), is developed and validated for the simultaneous determination of underivatised taurine and methionine in beverages rich in carbohydrates such as energy drinks. No initial clean-up procedure and no sample derivatisation are required. Satisfactory analysis was obtained on an Astec apHera NH_2 (150 mm × 4.6 mm; 5 μm) column with methanol–water (60/40) as mobile phase. The method was validated in terms of specificity, detection limits, linearity, accuracy, precision and stability, using threonine as internal standard. The potential effects of matrix and endogenous amino acid content were also examined. The limits of detection in the beverage varied from 20 μg L^{-1} for taurine to 50 μg L^{-1} for methionine.

Keywords: Underivatised amino acids; Hydrophilic interaction chromatography; Mass spectrometry; Taurine; Methionine; Assay validation

1. Introduction

Energy drinks and other dietary supplements are one of the most popular products used for competitive and recreational purposes by athletes in order to increase their sporting performances. These products not only quench thirst but also have many physiological and functional effects due to the fact that they contain compounds such as vitamins, carbohydrates and amino acids (AAs). It has been proven that there is a high correlation between the production and maintenance of muscle and the content of amino acids, especially that of taurine and methionine [1]. Taurine (2-aminoethanesulfonic acid) is a non-essential sulphur-containing amino acid which functions with glycine and γ-aminobutyric acid as a neuroinhibitory transmitter [2]. It has been shown to be essential in certain aspects of mammalian development [3] and exhibits various metabolic actions in the organism [4–7]. For all these reasons, taurine has been widely used as a stimulant additive in energy beverages. The synthesis of taurine in the body may depend on the availability of methionine. The latter is not synthesised in the body and must therefore be introduced from food sources or from dietary supplements. Methionine is also a powerful antioxidant and has proved to be a useful dissolving agent allowing the stabilisation of other amino acids in food products to prevent their oxidation [8]. Virtually all compound feeds are now supplemented with amino acids and the determination of these additives is an important quality assurance tool to assess the final product and monitor the production process.

Several methods for quantifying underivatised amino acids in carbohydrate-containing samples have been described in the literature. These methods include mainly the separation of amino acid and sugars by ion-exchange solid-phase extraction prior to HPLC analysis [9,10] and/or anion-exchange chromatography followed by integrated pulsed

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amperometric detection with specific waveforms [11–17]. However, these procedures have not gained wide acceptance due to baseline drift and potential instability.

For underivatised amino acid analysis, the detection of choice is mass spectrometry as this mode of detection has the advantage of providing additional structural information about the eluted compounds. Since 1995, the detection of underivatised AAs has been described by electrospray mass spectrometry (ESI-MS) showing the possibility of their positive as well as their negative ionisation [18–20]. Several complementary chromatographic systems are nowadays available for the separation of underivatised amino acids [21–23]. They use ion pair reversed-phase LC with volatile perfluorinated carboxylic acid as an ion pairing reagent. However, these chromatographic systems are not favourable for the direct quantification of taurine in a matrix rich in carbohydrates since, under these LC conditions, carbohydrates are eluted in void volume and simultaneously, a very weak retention is observed for taurine due to its high polarity. Afterwards, interferences from the matrix compounds eluted in the void volume area are observed even with a specific detection such as MS/MS.

Hydrophilic interaction chromatography (HILIC) may be an interesting alternative to reversed-phase liquid chromatography for the separation of such polar compounds. The HILIC mode was introduced by Alpert in 1990 [24] and later used in tandem with ESI-MS to separate and characterise polar low-molecular weight compounds such as amino acids, peptides, glycoconjugates and organic acids without derivatisation [25,26]. In the HILIC mode, an aqueous-organic mobile-phase (high organic content) is used with a polar stationary phase to provide normal-phase retention behaviour: silica and amino columns with aqueous mobile phases offer potential use in the HILIC mode [24,27]. This chromatographic mode is similar to normal phase chromatography since polar compounds are retained longer than non-polar ones and the polar mobile phase component (usually water) is the strong solvent.

The aim of our study was to develop a new LC–MS/MS method based on hydrophilic interaction chromatography (i) to exclude taurine and methionine from the void volume where ion suppression is maximal given that most of the matrix compounds are eluted in this area and (ii) to maintain selectivity among the two amino acids of interest and other interfering solutes of the matrices when tandem MS detection is not specific enough. The method was further applied for the simultaneous determination of underivatised taurine and methionine in an energy drink selected for its high carbohydrate content.

2. Experimental

2.1. Apparatus

The preliminary development of the chromatographic system was carried out with an LC-evaporative light scattering detection (ELSD) system. Chromatography was carried out using a Merck-Hitachi LaChrom system equipped with a L7100 quaternary pump (Merck, Darmstadt, Germany), a Rheodyne (Cotati, CA, USA) model 7125 injection valve fitted with a 10 μL loop, a Shimadzu (Kyoto, Japan) C-R6A integrator and a Sedere (Alfortville, France) model Sedex 55 ELSD set as follows: drift tube temperature, 50 °C; nebulizer gas pressure, 2.3 bar and photomultiplier, 9. ELSD has proved to be an inexpensive way to develop chromatographic methods that are directly transposable to LC–MS [21]. LC–ESI-MS/MS experiments were carried out using a Perkin-Elmer (Toronto, Canada) Model LC-200 binary pump, a Perkin-Elmer Sciex (Forster City, CA, USA) API 300 triple quadrupole mass spectrometer with a TurboIonSpray source heated at 350 °C. The mass spectrometer was operated in negative ion mode. Nitrogen was used as curtain and collision gas. After optimisation of the MS parameters, state and calibration parameters were as follows: nebulizer gas (NEB) = 10, curtain gas (CUR) = 7, collisionally activated dissociation (CAD) = 1, temperature (TEM) = 350 °C, ionspray voltage (IS) = −3800 V, declustering potential (DP) = −20 V, focusing potential (FP) = −200 V, entrance potential (EP) = −10 V, collision cell exit potential (CXP) = −15, ion energy 1 (IE1) = 1 eV, ion energy 2 (IE2) = 3 eV. Collision energy (CE) values varied from 10 to 15 eV depending on the mass transition monitored. The 10-nebulizer gas value corresponds to a flow-rate of 1.36 L min^{-1} and the 7-curtain gas value corresponds to a flow-rate of 1.02 L min^{-1}. The selective reaction monitoring (SRM) mode was used to monitor the parent and product ions for the tandem MS analysis of amino acids. The dwell-time was set at 200 ms and the pause time was 5.0 ms. Injections were done with a Perkin-Elmer series 200 autosampler fitted with a 10 μL loop. A Harvard Model 22 syringe pump was used for the optimisation of the MS parameters by infusing each amino acid solution (5 mg L^{-1} diluted in mobile phase) in the MS system at a flow-rate of 10 μL min^{-1}. All results were acquired with the Analyst version 1.3.1 software (Sciex Applied Biosystems).

Separation was carried out on an Astec apHera^{TM} NH_2 polymer 150 mm × 4.6 mm I.D., 5 μm (Whippany, NJ, USA) column. Flow-rate was 0.6 mL min^{-1}. For LC–MS/MS, the eluent from the column was split 1/6 before coupling to the mass spectrometer. The hold-up time was determined by injecting an aqueous solution of glucose (not retained under the optimised chromatographic conditions).

Column temperature was regulated by a Gecko-cil (Cluzeau, France) oven at 37 °C.

2.2. Chemicals and reagents

HPLC-grade methanol (MeOH) and acetonitrile (ACN) were obtained from J.T. Baker (Noisy le Sec, France).

Taurine (Tau), Methionine (Met) and Threonine (Thr) were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France). Deionised (18 MΩ) water, purified using

an Elgastat UHQ II system (Elga, Antony, France), was used for the preparation of analyte and mobile phase solutions.

2.3. Preparation of standard and quality control samples

Stock standard solutions (1000 mg L^{-1}) of Tau, Met and Thr were prepared by weighting and dissolving the adequate amount of each amino acid in deionised water and stored at 4 °C for a maximum period of 1 month. From the primary stock solution an intermediate stock solution (100 mg L^{-1}) was prepared in water. Seven calibration solutions (from 0.1 to 10 mg L^{-1}) were prepared by diluting the corresponding intermediate stock standard solution in mobile phase (MeOH/H_2O, 60:40, v/v). To simulate the matrix, the amino acid free beverage (blank beverage) was added to each calibration solution at the dilution 1/50. To establish the calibration curves and the quality controls within and between batches, threonine was used as internal standard and added to the different calibration solutions at a constant concentration (3 mg L^{-1}).

2.4. Sample preparation

The commercial energy drink and the corresponding amino acid-free sample were obtained from Taiwan. The sample was diluted (1/10 for LC-DEDL system or 1/50 for LC–MS/MS system) with the mobile phase (MeOH/H_2O, 60:40, v/v), spiked with threonine as internal standard for quantification and analysed without further pre-treatment.

2.5. Method validation

The method was validated according to the guidelines laid down by the US Food and Drug Administration (FDA) for bioanalytical method validation [28]. All results were expressed as percentages, where *n* represents the number of values or measurements. For the statistical analysis [29], Excel 2000 (Microsoft Office) was used. A 5% level of significance was selected.

Triplicate sets of calibration standard and quality control (QC) samples were analysed on 3 different days to determine the intra- and inter-day validations.

2.5.1. Limits of detection (LODs) and quantification (LOQs)

The LOD and LOQ were established for taurine and methionine using a blank sample spiked with taurine and methionine. Signal-to-noise ratios of approximately 3:1 and 10:1 were used for estimating the LOD and LOQ, respectively.

2.5.2. Specificity

Acceptable specificity was defined as the absence of any detectable SRM ion currents in LC–MS–MS, at the retention time of taurine, methionine or threonine (I.S.) in blank beverage sample.

2.5.3. Linearity

The calibration range for taurine and methionine was established using a triplicate set of standards (0.1, 0.25, 0.5, 1, 2.5, 5 and 10 mg L^{-1}) in blank beverage. The internal ratios (taurine or methionine peak area versus I.S. peak area) were calculated for each point and standard curves were constructed using least square linear regression analysis of internal ratios over concentration ratios. In order to check the homogeneity of the response variances, a Cochran test *C* was applied. The linear regression is weighted by $1/x$ (the reciprocal of the concentration ratio). F_1 and F_2 statistical tests were used to assess the linear model.

2.5.4. Accuracy and precision

According to the FDA guidelines, the minimum acceptance criterion for the intra- and inter-batch mean accuracies (% bias) of the calibration standards is within ±15% of the expected concentration (20% at the LOQ value). The intra- and inter-batch precisions (CVs) of the quality control samples should fall within ±15% (20% at the LOQ value). Three levels of concentration (0.25, 5 and 8 mg L^{-1}) were used to make the low, median and high level QC concentrations.

2.5.5. Stability

The stability of taurine and methionine in blank matrix was investigated by analysing triplicate QC samples at 0.25 and 10 mg L^{-1}. Freeze–thaw stability was determined after three cycles of freezing at −20 °C and thawing at room temperature. To assess short-term stability, the QC samples were thawed and left to stand at room temperature for 7 h. The long-term stability was also evaluated after 1 h, 1 week and 1 month at −10 °C. Stability criteria were as follows: if the mean signal decreases by no more than 15% (corresponding to the accepted imprecision of the assay), the analytes can be considered as stable.

3. Results and discussion

Tandem mass spectrometry was used as a sensitive and selective detection for the determination of taurine and methionine in energy drinks. Because a significantly lower response in electrospray positive ion mode than in negative ion mode has been claimed for taurine in several papers [20,21,30–32], it is preferable that a compatible LC system with a negative ionization mode be developed. The mass spectrometer was operated in negative ESI mode and the analytes were monitored in SRM mode using their most sensitive transition from their $[M-1]^-$ pseudomolecular ion as reported previously [20]: 124 > 80 for taurine; 148 > 47 for methionine and 118 > 74 for threonine.

3.1. Development of the HILIC method

HILIC provides separation of polar analytes and generally gives an elution order opposite to RPLC because the

stationary phase is more hydrophilic than the mobile phase. Solutes retained by the HILIC column are eluted by increasing the proportion of water or, in general, by increasing the polarity of the mobile phase. According to Alpert [24], selective partitioning of the analytes occurs between the (hydrophobic) mobile phase and the water-enriched layer on the surface of the stationary phase, which is involved in the separation mechanism of HILIC. To verify this partitioning mechanism employed effectively for the separation of carbohydrates [31] or peptides [24,27], the effects of organic modifier (methanol or acetonitrile) on the retention of taurine, methionine and mono-, di- or trisaccharides (glucose, saccharose and raffinose) were first studied using a polyamine bonded polymeric gel column (Astec NH_2). The results demonstrated that, in the absence of buffer, a significant normal-phase-type adsorption had occurred. In fact, amino acid retention time increases with the polarity of the solvent (the retention of taurine and methionine decreases from water > MeOH > ACN) and with decreasing organic modifier (MeOH or ACN) concentration in an aqueous mobile phase (data not shown). This elution pattern is in good agreement with the trends previously observed by Alpert for peptides and neutral carbohydrates [24]. Although methionine, taurine and small carbohydrates can be satisfactorily resolved whatever the organic modifier chosen (methanol or acetonitrile), the use of methanol allows a better reproducibility of the results, due to the small retention factor variations thereby obtained. Consequently, methanol was preferred and selected for our study. After optimisation, a mobile phase composed of MeOH/water (60:40, v/v) was found to provide the best result in selectivity and in total analysis time.

Fig. 1 shows the LC-ELSD chromatograms obtained under isocratic elution mode for (a) a beverage containing taurine and methionine and (b) a blank beverage without any amino acids. In order to detect simultaneously all the components present in solution, this analysis was carried out with an evaporative light scattering detector (ELSD). Under these chromatographic conditions, methionine and taurine were eluted with a retention time of 5.6 and 11 min, respectively. As can be seen in Fig. 1b, all the carbohydrates present in the drink (e.g. glucose, fructose, saccharose) are eluted in void volume and none of compounds present in blank beverage are eluted near the retention volume of taurine and methionine. The latter observation leads us to conclude there is a low risk of observing any interference between matrix components (e.g. carbohydrates or others) and the analytes of interest (e.g. methionine and taurine).

3.2. Choice of internal standard

The development of the quantification of taurine and methionine in beverages has been performed by using an internal standard methodology to correct the MS signal variations, commonly observed when the mass spectrometer is used for a long time. As is well-known, the most effective internal standards used in LC–MS(/MS) are stable isotopically labelled analogues of the compounds [23]. Since their properties in terms of chromatographic behaviour and ionisation are similar to those of the corresponding analytes, these substances enable the accuracy of the quantitative method to be increased. However, these substances are very costly, and beyond the financial means of most small laboratories.

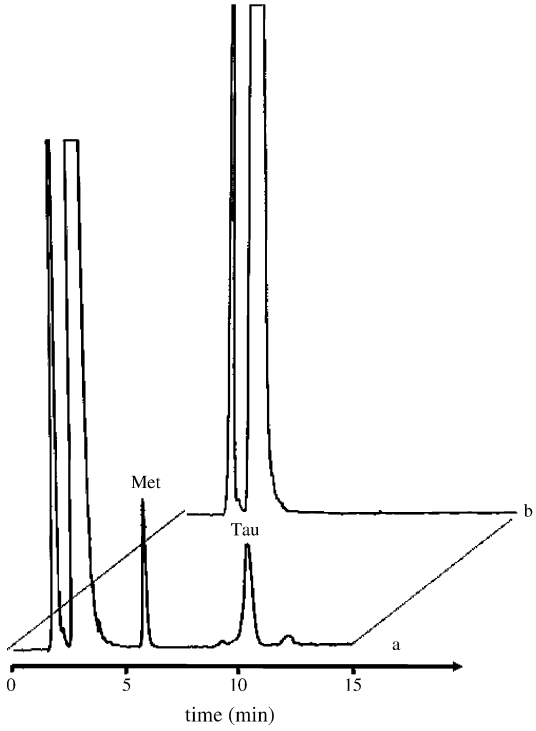

Fig. 1. HILIC–ELSD analysis of a beverage diluted to 1/10 with mobile phase. (a) Beverage containing Met and Tau, (b) amino acid-free beverage. Column: apHera NH_2 (150 mm × 4.6 mm I.D., 5 μm). Column temperature is at 37 °C. Mobile phase: MeOH/H_2O (60:40, v/v) under isocratic elution mode; flow-rate 0.6 mL min^{-1}; injection volume: 10 μL.

An alternative was found by the use of another amino acid (threonine) as internal standard. This amino acid was chosen due to its retention time (comprised between the retention times of taurine and methionine) and its response factor which is similar to that of methionine and taurine under the analytical conditions adopted. Moreover, no trace of this substance was found in the matrix analysed or in the blank beverage used for calibration by selecting its specific mass transition (118 > 74). From these observations, it has been concluded that threonine fulfils all the criteria required for monitoring taurine and methionine.

3.3. Assay validation

3.3.1. Evaluation of matrix effect and endogenous concentration of amino acids by using Student t-tests

The ionisation of analytes in the electrospray interface is influenced by various factors, in particular by the sample matrix. As a result, the response observed with MS/MS detection for a given concentration of analyte can change in the presence of other sample components. This change, referred

Table 1
MS/MS transitions, retention times and calibration parameters for methionine and taurine

Amino acid	Retention time (min)	LOD ($\mu g\,L^{-1}$)	LOQ ($\mu g\,L^{-1}$)	Calibration curve					
				Equation of linearity for $n=3$	r^2	t^a	C_c	F_1	F_2
Methionine	5.6	50	100	$y = 0.122x - 0.0005$ in matrix	0.9986	31.997	0.35	147945.45	2.88
		30	90	$y = 0.148x + 0.0001$ in mobile phase	0.9998				
Taurine	11	20	100	$y = 1.622x + 0.1414$ in matrix	0,9980	3.152	0.88	6384.23	1.423
		20	120	$y = 1.4805x + 0.1810$ in mobile phase	0.9979				

S_{a_1} and S_{a_2} are the corresponding standard deviations. These values were then compared with the theoretical t-value (=2.021), determined from a Student table for a 95% confidence interval.

[a] Calculated t-values were determined by applying the formula $t = |a_1 - a_2|/\sqrt{S_{a_1}^2 + S_{a_2}^2}$ where a_1 and a_2 are the slopes of the calibration curves prepared in mobile phase and in matrix, respectively.

to as ionisation suppression, generally involves a lack of necessary accuracy, precision and sometimes a loss of response for the analyte studied.

One experiment that can provide some information about the importance of matrix ion suppression is to compare the slope of a calibration curve of analyte prepared in a solution taken to be free from interference, such as the HPLC mobile phase, with the slope of a calibration curve constructed in a matrix blank spiked in the same concentration range of analyte. Comparing these two slopes shows the difference in response, assumed to be caused by components present in the matrix.

The comparison of calibration curve slopes was performed by applying the Student t-test for taurine and methionine. The obtained values (see Table 1) were significantly different since calculated t-values for methionine and taurine (=31.997 and 3.152, respectively) were above the theoretical value (=2.021), suggesting that interferences with other matrix components occurred during the detection step.

This type of information is very important for making decisions about the preparation of standard calibration solutions and means that calibration must be effected directly in a matrix blank spiked with a known concentration of analytes and internal standard.

The potential presence of an endogenous concentration of amino acids in the matrix blank was also tested by using the same calibration curves employed for the evaluation of matrix effect. After statistical data treatment of the y-intercept values by a second Student t-test, the values obtained were not significantly different (calculated t-values below the theoretical value): t-value for methionine was 0.005 and t-value for methionine was 0.008, compared to the theoretical value of 2.021. These results demonstrated that the effect of an endogenous contribution from blank beverage on the measurement of taurine and methionine is negligible.

3.3.2. Specificity

In this study, the specificity of the assay was established by simultaneously monitoring the deprotonated molecular ion and the major product ion derived from the parent ion for each amino acid of interest. Moreover, the choice of a negative ionisation mode rather than a positive ion mode for detecting taurine and methionine is more likely to increase the specificity since the organic molecules in general are better detected in positive ionisation mode [18].

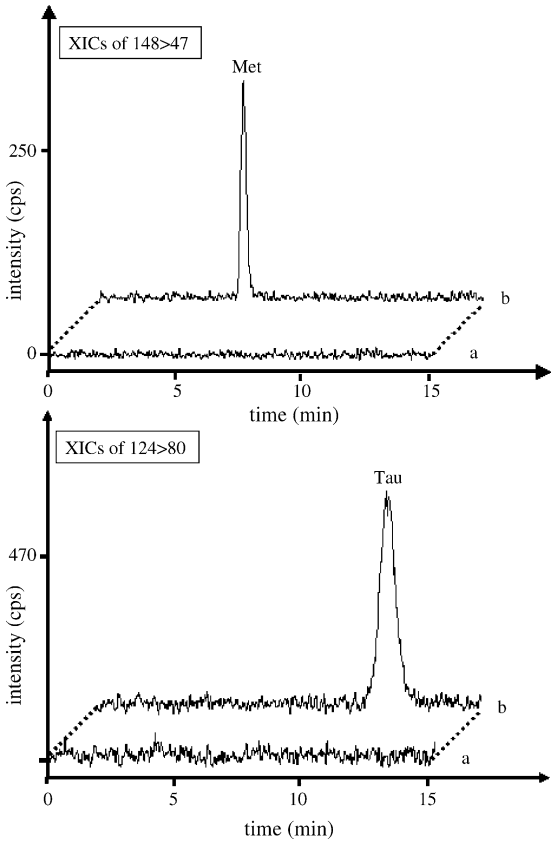

Fig. 2. LC–MS/MS chromatograms (specific extracted ion current) analysis for taurine or methionine of (a) blank beverage, (b) blank beverage spiked with 20 $\mu g\,L^{-1}$ of taurine or 50 $\mu g\,L^{-1}$ of methionine (corresponding LOD for each AA).

As illustrated in Fig. 2, no trace of interference substance was found at the same retention time as taurine and methionine by analysing the matrix blank.

3.3.3. Estimation of the limits of detection (LODs) and quantification (LOQs)

Limits of detection (LODs) and limits of quantification (LOQs) were evaluated for quantitative purposes (Table 1). From the values of the peak area ratios between compounds

and I.S., LODs of taurine and methionine were found to be about 20 and 50 $\mu g\,L^{-1}$, respectively when the standards are prepared in matrix. The LOQs measured for taurine, as well as for methionine, are equal to 100 $\mu g\,L^{-1}$, as reported in Table 1. With regard to the high content of expected amino acids in the energy drink analysed, these LOQ values were considered adequate to quantify taurine and methionine with suitable accuracy and precision.

3.3.4. Relationship between the response and the concentration

The linearity of an analytical method is defined by the FDA as the ability to obtain test results within a given range that are directly proportional to the concentration or amount of analyte in a sample. It has been determined at seven evenly spaced concentrations ranging from 0.1 $mg\,L^{-1}$ (lower limit of quantification) to 10 $mg\,L^{-1}$ (upper limit of quantification).

To evaluate the linearity, a statistical approach has been adopted in order to establish the most suitable regression model [29]. The statistical test set comprises homogeneity of variance by Cochran's test (C) and validity of the linear regression model by Fisher tests (F_1 and F_2).

The calculated value (C_c) was below the theoretical value for methionine but not for taurine. This result demonstrates that variance can be considered as constant for methionine and inhomogeneous for taurine for a 0.05 α-risk level (Table 1). Consequently, a weighting by $1/x$ is required for the calibration of taurine. In order to present a homogeneous data treatment, a weighting by $1/x$ has been also adopted for the calibration of methionine. The equations of linearity obtained are listed in Table 1. The determination coefficients (r^2) were higher than 0.998 for taurine and methionine.

The validity of the linear regression model was then assessed by using the Fisher tests F_1 and F_2. As can be seen in Table 1, the F_1-values for the linearity test were higher than the critical value for taurine and methionine and hence it can be concluded that there is a linear dependence between the response and the concentration of the analyte for a 0.05 α-risk level.

The F_2-test was also performed in order to validate the adaptation by a linear model regression. As F_2-values were higher than the critical value for taurine and methionine, it can be concluded that the linear model weighted by $1/x$ is suitable for the adopted response function.

3.3.5. Accuracy and precision

Intra- and inter-batch precision and accuracy were all measured with quality control samples containing both taurine and methionine at three concentration levels (0.25, 5 and 8 $mg\,L^{-1}$) and prepared in the same manner as the calibration samples in a matrix blank.

The accuracy of the assay was determined for taurine and methionine by comparing the nominal concentrations with the corresponding calculated ones. The minimum acceptance criterion for the accuracies (% bias) of the quality control

Table 2
Accuracy and precision from independently prepared QC samples

Amino acid	Nominal concentration ($mg\,L^{-1}$)	Intra-batch			Inter-batch		
		Concentration found ($mg\,L^{-1}$) for $n=3$	Accuracy (%) for $n=3$	Precision (% CV) for $n=3$	Concentration found ($mg\,L^{-1}$) for $n=3$	Accuracy (%) for $n=3$	Precision (% CV) for $n=3$
Methionine	0.25	0.3	101.2	2.5	0.2	97.6	3.3
	5	4.9	98.3	1.7	4.9	98.3	1.9
	8	8.1	101.7	1.4	8.3	103.9	2.2
Taurine	0.25	0.3	100.8	4.1	0.2	97.2	3.3
	5	5.0	100.8	2.1	4.9	98.6	2.1
	8	8.0	100.1	1.6	7.9	98.6	1.2

standards are within ±15% of the expected concentration (20% at the lower point of concentration). As reported in Table 2, very satisfactory accuracy was obtained for the three concentrations tested.

The intra-batch precision was examined by repeating the analysis of the same quality control samples three times under constant operating conditions. Table 2 shows the results obtained for taurine and methionine. The coefficient of variation (CV%) values indicate that for taurine and methionine there was little variation between the three series of measurements. The CVs obtained were in the range from 1.4 to 2.5% for methionine and from 1.6 and 4.1% for taurine.

The inter-batch precision was determined by analysing different quality control samples three times on 3 days. The CVs (%) obtained were always below 3.3 for methionine and taurine.

3.3.6. Stability of taurine and methionine in the matrix

Stability studies of taurine and methionine were carried out in analyte-free matrix in accordance with the guidelines of the Food and Drug Administration (FDA) [28]. The stability of the analytes was evaluated under conditions reflecting situations likely to be encountered during actual sample handling and analysis: stability after three freeze and thaw cycles, short-term stability (7 h) at room temperature (25 °C), long-term stability (after 1 day, 1 week and 1 month) at −10 °C. These stability procedures have been determined at three aliquots of each of the low and high concentration levels (0.25 and 10 mg L^{-1}, respectively). An evaluation of taurine and methionine stabilities in stock solution (1000 mg L^{-1}) stored in a freezer at −10 °C was also investigated.

As reported in Table 3, taurine and methionine were found to be very stable under the experimental conditions used for their analysis with a CV varying only from 0.6 to 6.5%.

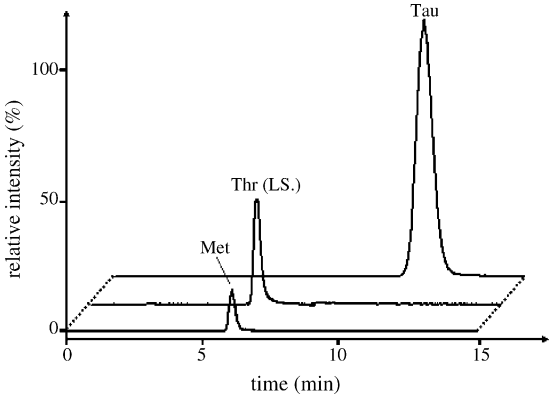

Fig. 3. HILIC-ESI-MS/MS analysis of a beverage diluted to 1/50 with mobile phase. Column: Astec apHera NH_2 (150 mm × 4.6 mm I.D., 5 μm). Column temperature is at 37 °C. Mobile phase: MeOH/H_2O (60:40, v/v) under isocratic elution mode; flow-rate 0.6 mL min^{-1}; split 1/6; injection volume: 10 μL.

3.4. Application to the simultaneous determination of taurine and methionine in an energy drink

The sample was analysed without pre-treatment except dilution at 1/50 with the mobile phase.

The method was further applied for the determination of taurine and methionine in an energy drink selected for its high carbohydrate content. A preliminary quantification of taurine and methionine showed that the concentrations of the two amino acids were sufficient to permit a dilution to 1/50 of the raw sample with the mobile phase.

Table 3
Stability determinations

Amino acid	Nominal concentration (mg L^{-1})	Stability determinations	Percentage of initial value (%)
Methionine	0.25	Freeze and thaw stability	89.3 ± 2.6
		Short-term stability at R.T.	100.0 ± 2.5
		Stability after 1 day at −10 °C	98.3 ± 2.0
		Stability after 1 week at −10 °C	95.9 ± 3.6
		Stability after 1 month at −10 °C	90.8 ± 6.5
	10.0	Freeze and thaw stability	95.0 ± 2.1
		Short-term stability at R.T.	102.6 ± 0.6
		Stability after 1 day at −10 °C	100.1 ± 3.4
		Stability after 1 week at −10 °C	95.8 ± 1.1
		Stability after 1 month at −10 °C	91.7 ± 1.7
Taurine	0.25	Freeze and thaw stability	98.3 ± 0.6
		Short-term stability at R.T.	99.1 ± 3.1
		Stability after 1 day at −10 °C	99.1 ± 3.1
		Stability after 1 week at −10 °C	98.7 ± 2.8
		Stability after 1 month at −10 °C	92.8 ± 4.6
	10.0	Freeze and thaw stability	97.3 ± 2.1
		Short-term stability at R.T.	98.3 ± 1.9
		Stability after 1 day at −10 °C	98.3 ± 1.9
		Stability after 1 week at −10 °C	96.6 ± 2.2
		Stability after 1 month at −10 °C	95.1 ± 1.7

This presents several advantages: (i) pre-concentration or other treatment steps prior to LC–MS/MS analysis can be avoided, (ii) the matrix effect and therefore ion suppression is considerably reduced by diluting the studied sample, and finally, (iii) the risk of polluting the mass spectrometer is significantly decreased.

The results obtained show a relatively low level of taurine (450 mg L^{-1}) and methionine (130 mg L^{-1}) in comparison with high levels of carbohydrates (>2000 mg L^{-1}). As expected in negative ionisation mode, the SRM chromatograms obtained for methionine and taurine were extremely clear, meaning that the AAs can be unambiguously identified (see Fig. 3).

4. Conclusions

A new HILIC-ESI-LC–MS/MS method has been developed and validated for the determination of taurine and methionine in an energy drink containing high proportions of carbohydrates.

This isocratic method is specific, simple (without any derivatisation step), fast (less than 15 min per analysis) and reliable. Acceptable levels of sensitivity, accuracy and precision were achieved and the studied amino acids show very good stability during the different handling treatments.

Finally, the proposed method has proven to be a valid alternative for detecting and quantifying taurine and methionine simultaneously in a carbohydrate-rich beverage. The contents of taurine and methionine in the analysed energy drink were estimated at 450 and 130 mg L^{-1}, respectively.

Future developments of this work will be the improvement of limits of detection (by about an order of magnitude) through the use of narrow-bore columns and/or different make-up between the separation and detection stages. This methodology could be therefore applied to other types of samples such as wine, beer, plasma or urine where amino acids are present in lower concentrations.

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