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Analysis of Nine Food Additives in Red Wine by Ion-Suppression Reversed-Phase High-Performance Liquid Chromatography Using Trifluoroacetic Acid and Ammonium Acetate as Ion-Suppressors

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A reversed-phase high-performance liquid chromatography (RP-HPLC) method was developed for the simultaneous determination of nine food additives, *i.e*., acesulfame, saccharin, caffeine, aspartame, benzoic acid, sorbic acid, stevioside, dehydroacetic acid and neotame in red wine. The effects of ion-suppressors, *i.e*., trifluoroacetic acid (TFA) and ammonium acetate (AmAc) on retention behavior of nine food additives in RP-HPLC separation were discussed in detail. The relationships between retention factors of solutes and volume percent of ion-suppressors in the mobile-phase systems of acetonitrile–TFA aqueous solution and acetonitrile–TFA–AmAc aqueous solution were quantitatively established, respectively. The results showed that the ion suppressors had not only an ion suppression effect, but also an organic modification effect on the acidic analytes. The baseline separation of nine food additives was completed by a gradient elution with acetonitrile–TFA(0.01%, v/v)–AmAc(2.5 mmol L^{-1}) aqueous solution as the mobile phase. The recoveries were between 80.2 – 99.5% for all analytes with RSDs in the range of 1.5 – 8.9%. The linearities were in the range of 0.2 – 100.0 mg L^{-1} with determination coefficients (r^2) higher than 0.9991 for all analytes. The limits of quantification (LOQs) were between 0.53 – 0.99 mg L^{-1}. The applicability of the proposed method to detect and quantify food additives has been demonstrated in the analysis of 30 real samples.

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Introduction

Recently, the role of food additives, *i.e*., preservatives (benzoic acid, sorbic acid and dehydroacetic), sweetener (acesulfame, saccharin, aspartame, stevioside, and neotame) and flavor agent (caffeine) has become more prominent with an increase in the production of processed and convenience foods.[1-6] As the most favorite food additives, they are limited by the national food safety standards in China. Among these standards, acesulfame, saccharin, caffeine, neotame, aspartame, stevioside, benzoic acid and dehydroacetic acid were prohibited to be added to the wine, and sorbic acid is regulated at the maximum concentration of 0.2 g kg^{-1}.[1,2] However, their presence at levels higher than the permitted safety levels can be harmful to human health. Some adverse effects, such as metabolic acidosis, convulsions, hyperpnoea, allergic reactions and bladder cancer in experimental animals and in humans, are described.[7,8] Therefore, developing an appropriate analytical method for the simultaneous and sensitive determination of the food additives mentioned above is essential.

A variety of analytical methods are available for the determination of food additives, such as UV spectroscopy, thin layer chromatography (TLC), reversed-phase high performance liquid chromatography (RP-HPLC) and gas chromatography (GC).[9-13] Spectroscopic methods are usually employed for the individual determination of these additives.[9,10] Because the additives can be present in combinations, chromatographic methods are often used for their selective individual or joint determination. Actually, most food additives have dissociation characteristics; therefore, some acids, bases, or buffers are added into the aqueous mobile phase in the RP-HPLC method according to the nature of analytes to suppress dissociation, which results in the improvements of the chromatographic retention behavior and peak shapes. However, RP-HPLC reports on the simultaneous determination of nine kinds of food additives, *i.e*., acesulfame, saccharin, caffeine, aspartame, benzoic acid, sorbic acid, stevioside, dehydroacetic acid and neotame (structural formulas and pK_a values are listed in Fig. 1), especially in food items are scarce. Wasik *et al*. reported a high-performance liquid chromatographic method with evaporative light-scattering detection (HPLC-ELSD), developed for the simultaneous determinations of multiple sweeteners, *i.e*., acesulfame, aspartame, neotame, saccharin *etc*., in soft drinks by using methanol–buffer solution–acetone (69:24:7, v/v/v) and methanol–buffer solution–acetone (11:82:7, v/v/v) as a mobile-phase system.[14] Techakriengkrai *et al*. reported a HPLC method for the rapid determination of benzoic acid and sorbic acid in Thai rice wine and distillate products by using 0.01 mol L^{-1} ammonium acetate buffer–methanol (60:40, v/v) as a mobile-phase system.[15] Therefore, developing an appropriate analytical method for the simultaneous determination of food additives *i.e*., multiple sweeteners, preservatives and flavor

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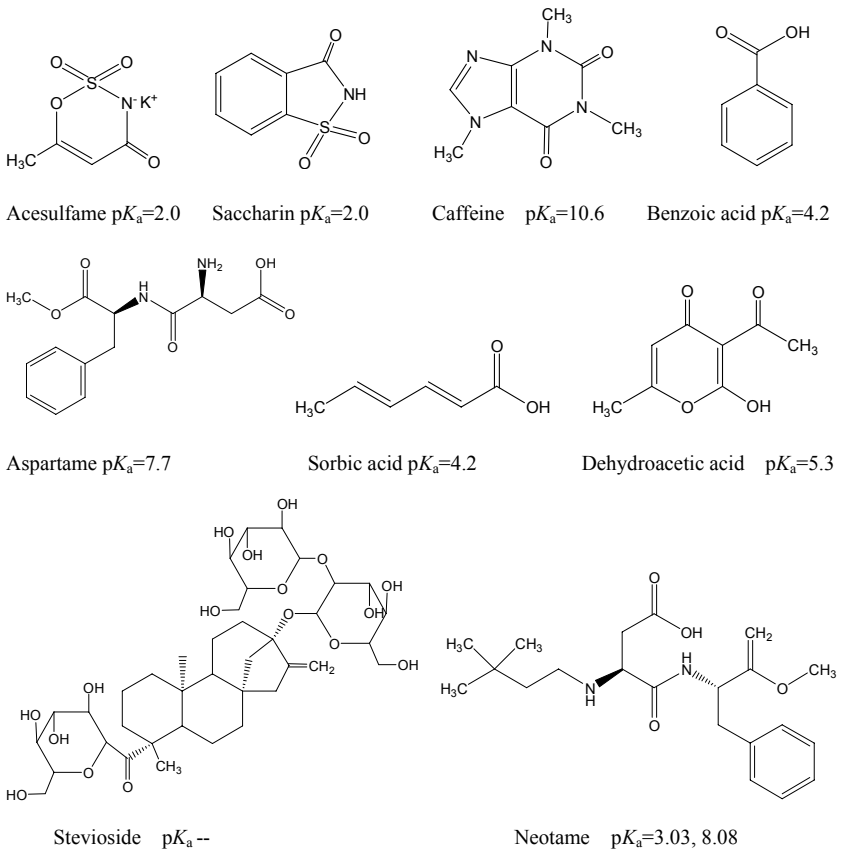

Fig. 1 Structural formulas and pK_a values of nine food additives.

agent, mentioned above, is necessary. Furthermore, although simple acids and buffers, have been widely applied to suppress the ionization of weakly ionizable acidic analytes in RP-HPLC,[16,17] none of the previously reported studies focused on systematic studies concerning the retention behavior of the food additives mentioned above in the ion-suppression RP-HPLC mode, which would be much useful to develop an analytical method for the simultaneous determinations of more food additives in food items.

The object of this study was to develop a rapid and efficient analytical method for the simultaneous determinations of nine food additives in red wine by HPLC with ultraviolet (UV) detection. Special attention was devoted to optimization of the chromatographic separation conditions by intensively investigating their retention behavior by using ammonium acetate (AmAc) and trifluoroacetic acid (TFA) as ion-suppressors.

Experimental

Reagents and materials

Acesulfame, saccharin, caffeine, aspartame, benzoic acid, sorbic acid, stevioside, dehydroacetic acid and neotame were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Acetonitrile, ammonium acetate (AmAc), trifluoroacetic acid (TFA) of HPLC grade were purchased from Merck Company (Darmstadt, Germany). The red wine samples were acquired in local markets (Ningbo, China).

Equipment

A vortex mixer, Hualida WH-866 (Taicang, China), was used during extraction. HPLC analysis was performed with an Agilent 1100 system equipped with a G1311A pump, a G1314A UV detector and a G1313B autosampler (Palo Alto, CA).

Chromatographic conditions

RP-HPLC analysis was performed on a Gemini C18 column (250 mm × 4.6 mm i.d. × 5 μm). Analytes were separated by RP-HPLC using 2.5 mmol L^{-1} AmAc and 0.01% TFA (v/v) in water as eluent (A), and acetonitrile as eluent (B). The linear gradient was: 0 – 10 min, 95 – 87% A (5 – 13% B); 10 – 15 min, 87 – 59% A (13 – 41% B); 15 – 30 min, 59 – 70% A (41 – 30% B). Finally, the mobile-phase concentration was returned to 95% A (5% B), and held for 10 min for column equilibration. Chromatographic separation of the food additives was accomplished at a constant flow of 1.0 mL min^{-1}. The column was thermostated at 30°C to increase the retention time reproducibility. The detection wavelength was set at 210 nm and the injection volume was 10.0 μL.

Sample preparation

A 10.0-mL red wine sample was placed into an open evaporating dish to heat for 30 min in a water bath at 50°C, and allowed to evaporate to a volume of approximately 5.0 mL. Afterwards, the solution was transferred to a 10.0-mL colorimetric cylinder, and deionized water was added to the mark of 10.0 mL, vortexed, and 1.0 mL aliquot of the supernatant was filtered using a 0.22-μm membrane prior to its injection into the RP-HPLC system.

Method validation

Individual stock standard solutions were prepared at the

1000 mg L^{-1} level by exact weighing and dissolution in water–methanol (1:1, v/v); these solutions were stored under refrigeration ($T \leq 4^{\circ}C$). Stock mixture solutions of the standards at a concentration of 100.0 mg L^{-1} were prepared by appropriate dilution of the stock solutions with water–methanol (1:1, v/v). Calibration standards in acetonitrile with concentration in the range of 0.2 – 100.0 mg L^{-1} were also prepared before use for calibration curves. The calibration curves made by peak area *vs*. concentration (mg L^{-1}) were used to calibrate the RP-HPLC system, and to spike samples in recovery experiments.

Spiked recoveries were performed at concentrations of 1.0, 10.0 and 20.0 mg L^{-1} for 9 food additives in the samples. For each spiked sample, a stock mixture solution of the standards was added to 10.0 mL red wine, which was free from the target compounds. The spiked samples prepared were stored at 4°C for about 12 h to let the food additives permeate uniformly into the samples. Five recoveries at each level were run along with both a reagent and a sample blank.

Results and Discussion

Effect of AmAc on chromatographic separation

The aim of our research was to develop a simple, accurate and reliable method that would allow the determination of nine food additives in red wine at the same time. In our willingness, the method not necessarily should have to show better performances than the single methods for a single food additive, but should have to provide a versatile tool for red wine quality-control purposes, for all of the food additives at the same time, allowing a more powerful screening potential, especially for control agencies.

In order to obtain optimization of chromatographic separation, the organic modification effects of ion suppressors, *i.e*., AmAc on the chromatographic separation, was studied, which would result in an improvement of the chromatographic retention behavior and peak shapes of nine food additives. The stock AmAc solution was prepared at 0.2 mol L^{-1} level by exact weighing and dissolution in water; the solution was stored under refrigeration ($T = 4^{\circ}C$). A series of aqueous mobile phases were composed of different concentrations of AmAc prepared at 2.5, 5.0, 10.0, 15.0 and 20.0 mmol L^{-1} levels by appropriate dilution of the stock AmAc solution with water, respectively. The results are shown in Fig. 2. It can be seen that the retention time of benzoic acid, acesulfame, dehydroacetic acid, sorbic acid, saccharin and neotame decreased from 12.50 to 7.11 min, 13.51 to 7.56 min, 13.78 to 8.02 min, 14.13 to 8.02 min, 16.94 to 10.26 min, and 20.70 to 20.39 min, respectively, with an increase of the concentration of AmAc from 2.5 to 15.0 mmol L^{-1}. The chromatographic retention behavior of the 6 food additives mentioned above can be described by Eq. (1) regarding the retention factor (k) with the volume fraction of an organic modifier (C_B) in the binary hydro-organic mobile phase for liquid chromatography,[18,19] reflecting the organic modification effects of AmAc in the mobile-phase system of AmAc solutions as eluent (A) and acetonitrile as eluent (B).

$$\ln k = a + cC_B \quad (1)$$

The k value was calculated according to the equation $k = (t_R - t_0)/t_0$, where t_0 was determined by using $NaNO_3$ as the holdup time marker. Parameters a and c have their own definite physico-chemical meanings. Parameter a, the natural logarithm of the hypothetical k_w, corresponding to neat aqueous fraction of the mobile phase, is related to the hydrophobicity of a solute on the stationary phase. Parameter c, the regression coefficient of C_B, is an entropy function of an adsorbed solute and a constant for a given solute-eluent combination, reflecting the interaction between analytes and eluent molecules.

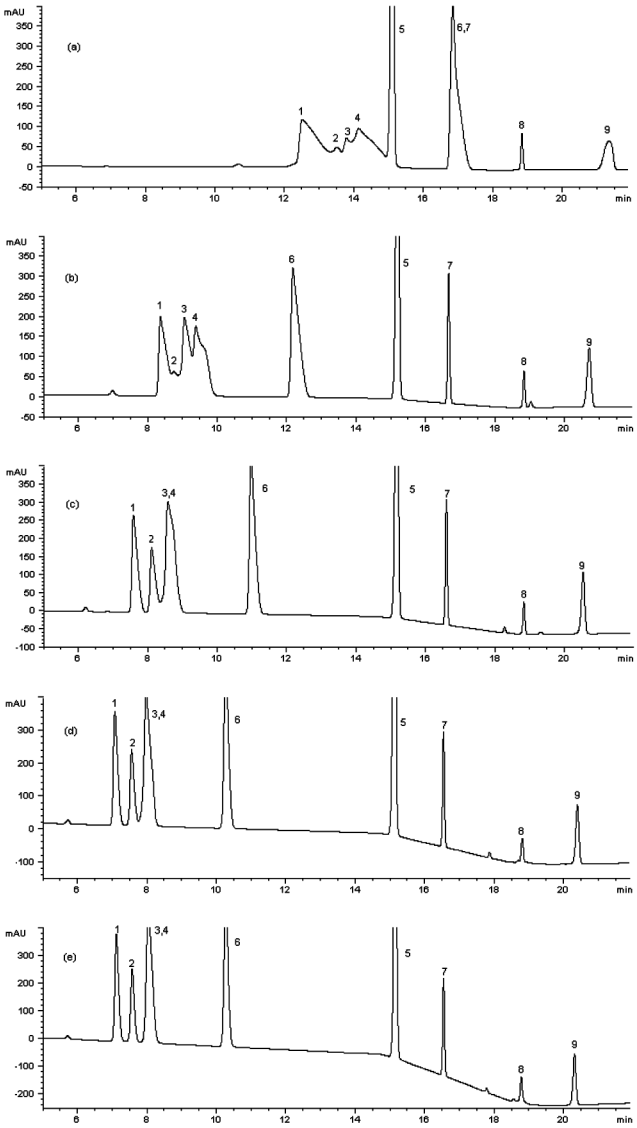

Fig. 2 Chromatograms of 9 food additives in five mobile-phase system: (a) acetonitrile–AmAc (2.5 mmol L^{-1}), (b) acetonitrile–AmAc (5.0 mmol L^{-1}), (c) acetonitrile–AmAc (10.0 mmol L^{-1}), (d) acetonitrile–AmAc (15.0 mmol L^{-1}) and (e) acetonitrile–AmAc (20.0 mmol L^{-1}) with peak numbering: benzoic acid (1), acesulfame (2), dehydroacetic acid (3), sorbic acid (4), caffeine (5), saccharin (6), aspartame (7), stevioside (8) and neotame (9).

The results are listed in Table 1. It can be seen that the ln k of benzoic acid, acesulfame, dehydroacetic acid, sorbic acid, saccharin and neotame with the corresponding C_{AmAc} can be approximately fitted to linear equations with determination coefficients (r^2) higher than 0.9686, depending on AmAc used. The good r^2 values here reveal that the ion suppressor of AmAc not only has an ion-suppression effect, but also an organic modification effect on benzoic acid, acesulfame, dehydroacetic acid, sorbic acid, saccharin and neotame in the mobile-phase system of AmAc solutions as eluent (A) and acetonitrile as eluent (B).

Effect of TFA on chromatographic separation

In the mobile-phase system of an acetonitrile–AmAc aqueous solution, though the chromatographic retention and peak shape of 9 food additives had been improved, dehydroacetic acid and sorbic acid had not obtained baseline separation. Thus, a series of aqueous mobile phases were composed of AmAc (2.5 mmol L^{-1}) and different concentrations of TFA prepared at 0.005, 0.008, 0.010, 0.012 and 0.015% (v/v) levels by appropriate dilution of the TFA solution with water, respectively, were used for further improvement of the chromatographic retention and the peak shape of the 9 food additives. Also the results showed that the retention times of benzoic acid, sorbic acid and dehydroacetic acid were increased from 15.16 to 17.08 min, 17.42 to 18.50 min, and 19.33 to 20.33 min, respectively, with increasing of the concentration of TFA from 0.005 to 0.015% (v/v). In the aqueous mobile phase, the pH value was in the range of 2.5 – 3.3, and so the ionized ability of weak acidic compounds ($7.0 > pK_a > 3.5$), *i.e.*, benzoic acid, sorbic acid and dehydroacetic acid, was greatly suppressed by TFA in the aqueous mobile phase. This would cause the analytes retention times to be delayed and the trends would be obvious with the decreasing of pH value (the increasing of the concentration of TFA). However, in the case of acesulfame and saccharin ($pK_a = 2.0$), with increasing of the concentration of TFA from 0.005 to 0.008% (v/v), their retention times were increased from 8.52 to 8.77 min and 11.54 to 11.83 min, respectively, and any further increase in the concentration of TFA led to a gradual decrease of the retention time to 7.31 and 10.09 min. This phenomenon can be explained that TFA was regarded not only as an ion suppressor (the ionized fraction), but also as an organic modifier (the unionized fraction). Under the higher pH value, the concentration of TFA was relatively lower in the mobile phase, while most of them were ionized, and so TFA in the mobile phase acted predominantly as an ion suppressor. However, under the lower pH value, the concentration of TFA was relatively higher in the mobile phase, and most of them existed in molecular form; TFA in the mobile phase acted predominantly as organic modifiers, and the relationships between the retention factors of acesulfame and saccharin and volume percent of TFA in the mobile phase system of acetonitrile–TFA–AmAc are shown in Table 2. The good r^2 values here reveal that the ion suppressor of TFA has not only an ion suppression effect, but also an organic modification effect on acesulfame and saccharin in the mobile-phase system of AmAc and TFA solutions as eluent (A) and acetonitrile as eluent (B). Moreover, all of the nine food additives had obtained baseline separation in the mobile-phase system of acetonitrile–TFA (0.010%, v/v)–AmAc (2.5 mmol L^{-1}) aqueous solution, as shown in Fig. 3.

Table 1 Relationships between the retention factors of acesulfame, saccharin, benzoic acid, sorbic acid, dehydroacetic acid, neotame and the volume percent of ammonium acetate in the mobile-phase system of acetonitrile–AmAc

Food additive	Acetonitrile–AmAc	
	Relation equation	r^2
Acesulfame	$\ln k = 0.821 - 29.17C_{AmAc}$	0.9903
Saccharin	$\ln k = 1.256 - 30.73C_{AmAc}$	0.9686
Benzoic acid	$\ln k = 0.777 - 35.05C_{AmAc}$	0.9999
Sorbic acid	$\ln k = 0.933 - 31.68C_{AmAc}$	0.9974
Dehydroacetic acid	$\ln k = 0.892 - 28.96C_{AmAc}$	0.9794
Neotame	$\ln k = 1.865 - 2.36C_{AmAc}$	1.000

Table 2 Relationships between the retention factors of acesulfame and saccharin and the volume percent of TFA in the mobile-phase system of acetonitrile–TFA–AmAc

Food additive	Relation equation	r^2
Acesulfame	$\ln k = 7.220 - 314.5C_{TFA}$	0.9996
Saccharin	$\ln k = 7.648 - 245.0C_{TFA}$	0.9991

Method linear range, accuracy, LOD and LOQ

The linearity of the calibration curves made by peak area *vs.* concentration (mg L^{-1}) was studied using calibration standards

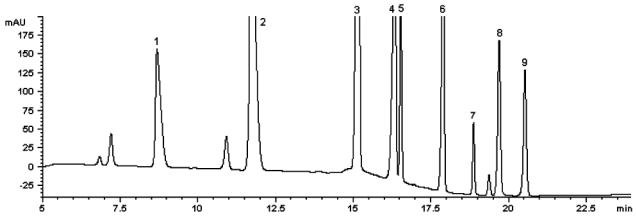

Fig. 3 Chromatograms of 9 food additives in the mobile-phase system: acetonitrile–TFA(0.010%, v/v)–AmAc(2.5 mmol L^{-1}) with peak numbering: acesulfame (1), saccharin (2), caffeine (3), benzoic acid (4), aspartame (5), sorbic acid (6), stevioside (7), dehydroacetic acid (8) and neotame (9).

Table 3 Validation parameters obtained for the 9 food additives at three concentration levels in red wine ($n = 9$)

Food additive	Retention time/min	Linear equation	Linearity range/ mg L^{-1}	1.0 mg L^{-1}		10.0 mg L^{-1}		20.0 mg L^{-1}		LOD/ mg L^{-1}	LOQ/ mg L^{-1}	r^2
				Average recovery, %	RSD, %	Average recovery, %	RSD, %	Average recovery, %	RSD, %			
Acesulfame	8.68	$y = 18.231x + 19.986$	0.5 – 50	95.6	2.6	95.9	3.6	94.8	2.6	0.19	0.63	0.9996
Saccharin	11.70	$y = 49.630x + 20.346$	0.5 – 50	92.3	5.6	93.8	3.9	93.5	5.1	0.22	0.73	0.9999
Caffeine	15.13	$y = 72.870x + 31.238$	0.2 – 20	90.6	5.0	92.6	2.7	94.1	3.6	0.16	0.53	0.9999
Benzoic acid	16.32	$y = 26.019x + 3.376$	0.5 – 50	90.2	4.4	91.7	3.3	91.9	4.2	0.23	0.76	0.9998
Aspartame	16.53	$y = 16.258x + 4.5037$	0.5 – 50	81.4	2.6	81.3	3.8	82.0	4.9	0.24	0.79	0.9995
Sorbic acid	17.88	$y = 22.555x + 3.7054$	0.5 – 50	98.1	3.6	95.9	2.8	95.0	4.0	0.20	0.66	0.9998
Stevioside	18.86	$y = 3.875x + 1.9635$	1.0 – 100	99.5	8.9	97.2	7.8	95.8	5.6	0.30	0.99	0.9991
Dehydroacetic acid	19.69	$y = 14.899x + 8.6486$	0.5 – 50	93.7	2.2	93.8	2.9	93.5	2.3	0.18	0.59	0.9993
Neotame	20.52	$y = 12.714x + 3.3612$	0.5 – 50	80.2	5.1	80.6	1.5	80.3	2.9	0.19	0.63	0.9991

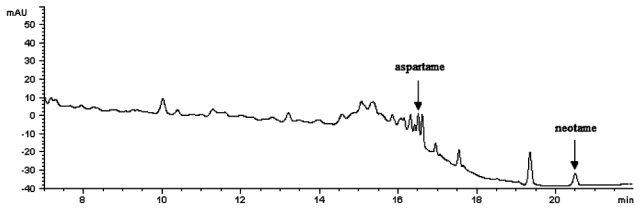

Fig. 4 RP-HPLC chromatogram for one of the detected red wine samples.

in solvent at seven concentrations of 0.5, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0 and 100.0 mg L^{-1}. The response function was found to be linear with a coefficient of determination (r^2) higher than 0.999 in the tested range listed in Table 3 for 9 food additives.

The method accuracy and the precision data were obtained by 9 food additives spiked at concentrations of 1.0, 10.0 and 20.0 mg L^{-1} in red wine. The results are summarized in Table 3. The mean recoveries were in the range of 80.2 – 99.5% at three spiked levels with relative standard deviations (RSDs) in the range of 1.5 – 8.9%.

The LODs and LOQs values for the analyzed food additives are shown in Table 3. It can be seen that the LODs and LOQs calculated as the lowest analyte concentration that yielded a signal-to-noise (*S*/*N*) ratio of 3 and 10 were in the range of 0.16 – 0.30 and 0.53 – 0.99 mg L^{-1}, respectively.

Real samples

Three kinds of red wine (ten samples for each kind), including Dry red wine, Demi-sec wine and Doux wine, were analyzed by the developed method. Each batch of samples was processed together with a matrix blank, which was obtained from a food additive-free sample. The matrix blank eliminated any false positive as the result of contamination in the extraction process, instrument or chemicals. A reagent blank was obtained by performing the whole process without a sample. This sample eliminated any possible false positives produced by contamination in the instrument or solvent used. A blank extract spiked at concentrations of 10.0 mg L^{-1} permitted was to control the extraction efficiency. Calibration curves were prepared daily, obtaining determination coefficients >0.999. The results show the presence of aspartame and neotame in three of the thirty collected samples with concentrations of 3.93 and 5.21 mg L^{-1}, respectively (Fig. 4); another 7 food additives were not found above LOQ in the analyzed samples.

Conclusions

In this work, an analytical RP-HPLC method for the determination of 9 food additives in red wine samples was developed. The effect of ion suppressors, *i.e*., AmAc and TFA, on the chromatographic separation has been intensively investigated. The results show that AmAc and TFA both have ion suppression and organic modification effects, which could result in improving the chromatographic retention and the peak shapes. Based on the RP-HPLC with UV detection, a single method was established for the simultaneous determination of 9 food additives in red wine by a gradient elution in the mobile-phase system of acetonitrile–TFA(0.01%, v/v)–AmAc (2.5 mmol L^{-1}) aqueous solution.

Acknowledgements

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