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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETECTION AND QUANTITATION OF AMINES IN MUST AND WINE

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SUMMARY

Three fluorigenic reagents were tried in order to increase the sensitivity of the detection of various amines. The derivatives formed were then used to develop a reversed-phase high-performance liquid chromatographic (HPLC) procedure for the separation of at least five amines. Dns-Cl and fluorescamine were rejected. The chromatogram of Dns-amines from red wine was overcrowded with unidentifiable peaks. It was then postulated that ammonia or phenol derivatives or other by-products of the Dns derivatization reaction interfered with the separation of amines. Fluorescamine, although it produced highly fluorescent derivatives, had the drawback of reacting with di- and polyamines to give more than one derivative and this interfered with the resolution.

o-Phthaldialdehyde (OPT) was used successfully for the derivatization of amines in red must and wine. The method involved the reaction of amines with OPT in the presence of mercaptoethanol followed by extraction of the derivatives with ethyl acetate. A reversed-phase HPLC system was developed for the separation of OPT derivatives of agmatine, cadaverine, ethanolamine, histamine, phenylethylamine, putrescine, tryptamine, tyramine, spermine and spermidine within 40 min.

INTRODUCTION

The method commonly proposed for the determination of histamine in must and wine is based on gravity cation-exchange extraction, derivatization of the fraction of interest with *o*-phthaldialdehyde (OPT), followed by measurement of the fluorescence[1]. This method is, however, not specific and interference by some closely related substances could occur. Other amines, *e.g.*, tyramine and putrescine, have been identified and semi-quantified using paper or thin-layer chromatography.

Automated ion-exchange chromatography, similar to that used for amino acid analysis, was adapted for the detection of histamine in wine by Mack[2], for the simultaneous determination of putrescine, histamine, cadaverine, 4-azaheptamethy-

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lenediamine, spermine, tyramine and phenylethylamine in wine by Woidich *et al.*[3] and for the detection of histamine, putrescine, cadaverine, tyramine and agmatine in beer by Zee *et al.*[4].

Battaglia and Frölich[5] published a method for the determination of histamine in wine as the Dns-Cl derivative, using normal-phase high-performance liquid chromatography (HPLC). They later improved this method to include the separation of more than twenty amines, of which ten were identified with certainty[6]. The stability of the various Dns-Cl complexes and the method of quantitation were not given.

Subden *et al.*[7] proposed a reversed-phase HPLC method for the quantitation of histamine in wines. In this instance, the OPT–histamine complex was not extracted from its original medium. The method of detection was not based on the fluorescence of the OPT–histamine condensation product but on its absorbance at 210 or 280 nm. This resulted in a decrease in sensitivity.

The objective of this study was to develop a reliable analytical procedure for the separation and quantitation of agmatine, cadaverine, ethanolamine, histamine, phenylethylamine, putrescine, tryptamine, spermine, spermidine and tyramine in must and wine. In order to achieve this objective, three derivatization reagents were investigated: Dns-Cl, fluorescamine and OPT.

MATERIALS AND METHODS

Apparatus for high-performance liquid chromatography

Two instruments were used. One consisted of a Waters Model 6000A solvent delivery device, a Waters U6K injector, a Waters Model 660 solvent programmer, a Waters Model 440 absorbance detector (Waters Assoc., Milford, MA, U.S.A.), a Spectra-Physics Model SP4000 central processor and a Model SP4020 data interface (Spectra-Physics, Santa Clara, CA, U.S.A.). The other was a Spectra-Physics Model SP8000B with the following features: the pump is a microprocessor-controlled stepper motor driving dual reciprocating pistons, a temperature-controlled column compartment, a manual injector with a loop capacity of 10 μl, a dual-channel printer/plotter, a helium de-gassing system, a ternary solvent system, a data system and a UV–visible absorbance detector (Spectra-Physics).

Detector

The detector used with both systems was a Waters Model 420 fluorimeter. The detector was equipped with an F4T5BL lamp; the gain was set at different levels of sensitivity depending on the method of derivatization. The following excitation and emission filters were used: with Dns-Cl, excitation at 360 nm band pass, emission at 460 nm long pass; with fluorescamine, excitation at 395 nm interference, emission at 460 nm long pass; and with OPT, excitation at 340 nm, emission at 440 nm.

Column

A μBondapak C_{18} column (30 cm × 3.9 mm I.D.) with a particle size of 10 μm (Waters Assoc.) was used for reversed-phase liquid chromatography (LC).

Aqueous solvents

The water used for the mobile phases was treated as follows: freshly doubly

distilled water was filtered through a 0.45-μm type HA membrane filter (Millipore, Bedford, MA, U.S.A.), passed through a μBondapak C_{18} column to remove trace amounts of organic materials and stored at −29°C until needed. Stocks of water were prepared every 15 days. Later the Norganic system provided by Millipore was used instead of the C_{18} column.

Solutions of acetic acid (0.08 *M* and 0.02 *M*) were prepared by adding the required amount of ACS-grade glacial acid to water prepared as above. The mixture was filtered through a 0.45-μm type HA membrane filter (Millipore) and divided in portions of 500 ml which were stored at −29°C. This solvent was prepared every 15 days.

Buffer solutions were prepared with ACS-grade chemicals and purified water and processed in the same manner as for acetic acid.

When the Waters LC system was used, the solvents were sonicated for 30 min to eliminate entrapped gas. With the Spectra-Physics LC system, a special feature of the system permitted a continuous purge of the solvents with helium gas.

Aqueous mobile phases were renewed every day.

Organic solvents

Methanol and acetonitrile were of HPLC grade (Fisher Scientific, Toronto, Canada). They were filtered through a 0.5-μm type FH membrane filter (Millipore) and sonicated or purged with helium gas as mentioned above for aqueous solvents. Organic solvents were stored at 20 ± 3°C.

Mobile phases

Various combinations of methanol, acetonitrile, acetic acid and buffers were used in order to optimize the separation and resolution of the amines. The mobile phases either remained stable during the chromatographic procedure (isocratic) or changed from a relatively polar to a non-polar composition with time. Combinations of isocratic and gradient elutions were also tried.

Chemicals

Fluorescamine, Dns-Cl, OPT, mercaptoethanol and amine standards were obtained from Sigma (St. Louis, MO, U.S.A.). Acetone, ethyl acetate and methanol were of HPLC grade (Fisher Scientific). All other chemicals met the ACS specifications. Stock solutions of amines in water (0.01 *M*) were prepared, filtered through a 0.45-μm type HA membrane filter (Millipore) and stored at 4°C. These stock solutions were further diluted as required to prepare calibration graphs. The water used for amine solutions or other reagent solutions was always prepared as described under *Aqueous solvents*.

Methods of derivatization

Three reagents for derivatization were investigated: Dns-Cl, fluorescamine and OPT.

Dns-Cl. To 1.0 ml of wine (or standard solution) were added in succession 3.0 ml sodium carbonate (4% in water) and 3 ml of Dns-Cl reagent (1% Dns-Cl in HPLC-grade acetone). The mixture was allowed to react in the dark at 40°C for 30 min. The acetone was evaporated with a stream of nitrogen and the derivatives were

extracted into 3.0 ml of ethyl acetate (modification of the method of Battaglia and Frölich[5]). The ethyl acetate phase was used for injection.

Fluorescamine. A 250-μl volume of sample (wine or standard) was placed in a 5-ml vial containing 250 μl of borate buffer (pH 8.0) (prepared by titrating 0.1 *M* boric acid with 6 *M* sodium hydroxide solution). While mixing the contents of the vial on a vortex mixer, 250 μl of the fluorescamine reagent (12.5 mg of fluorescamine in 25 ml of HPLC-grade acetone) were added and the stirring was continued for a further 30 sec. The mixture was then ready for injection (adaptation of the method of Samejima[8]).

o-Phthaldialdehyde (OPT). The method of derivatization is a modification of that of Davis *et al.*[9]. To 0.5 ml of an amine standard solution was added 1.0 ml of OPT reagent [20 ml of borate buffer (pH 10.40 ± 0.02), 180 mg of OPT, 2 ml of methanol and 0.4 ml of 2-mercaptoethanol]. The mixture was allowed to react in the dark for exactly 4 min. Immediately after, 2 g of sodium chloride were added, followed by 4 ml of ethyl acetate. The contents were mixed on a vortex mixer for exactly 1 min and the two phases were allowed to separate. The supernatant was used for injection into the HPLC system. If wine was the test material, 2 ml of wine were used instead of the standard solution.

Quantitation

Internal standard. Because tryptamine has not been reported to occur in wine, it was used as the internal standard (IS) in the preliminary development of the analytical procedure. The internal standard technique was used simultaneously with the method of external standards. The results obtained using peak areas and peak areas adjusted for the incorporation of an IS were compared.

External standards. Calibration graphs were obtained using standard solutions of amines. The minimum and maximum concentrations (amounts injected in 10 μl) for each amine were as follows: agmatine, 0.5–100 pmol; cadaverine, 6–120 pmol; ethanolamine, 13–1650 pmol; histamine, 5–1500 pmol; phenylethylamine, 7.5–150 pmol; putrescine, 5.5–100 pmol; tyramine, 5–1500 pmol; and spermidine, 5–100 pmol.

Because almost 1 h was required to complete the analysis of one sample (elution for 40 min plus time for equilibration of the column), a calibration graph was not prepared each working day. Instead, the following procedure was adopted. Three sets of data were obtained (set 1 had four replicates with six concentrations of an amine per replicate; set 2 had six replicates with two concentrations of an amine per replicate; set 3 had two replicates with six concentrations of an amine per replicate). A regression analysis was performed on each set of data. All three regressions proved to be linear and a test of homogeneity of slopes indicated that they were not different ($\alpha = 0.05$). Therefore, a weighted slope and its associated confidence limits were calculated. Every working day, only two concentrations of amine standards were injected and a slope obtained. If the confidence limits of the weighted slope encompassed the new standard slope, then it was concluded that the instrument was performing satisfactorily.

RESULTS AND DISCUSSION

Derivatization with Dns-Cl

Dns-Cl was considered as a derivatization agent because its derivatives have

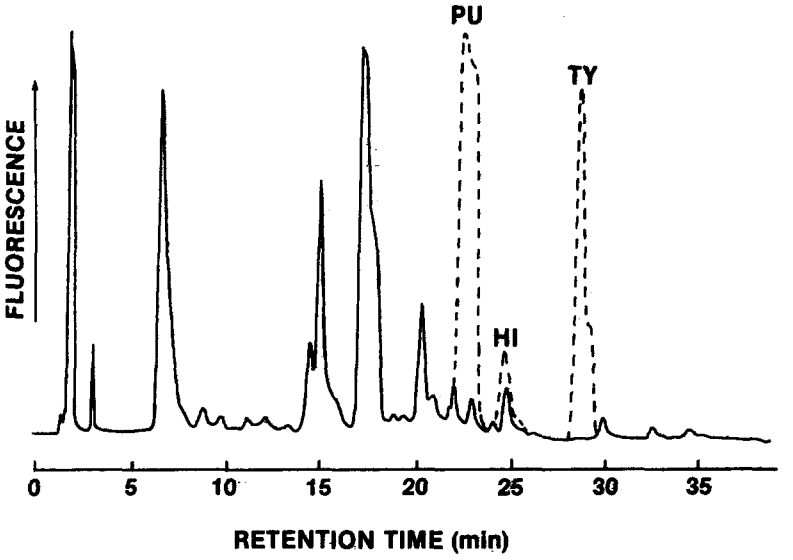

Fig. 1. Reversed-phase HPLC separation of histamine, tyramine and putrescine from Villard Noir wine derivatized with Dns-Cl. Instrument: Waters Assoc. Column: 30 cm × 3.9 mm I.D., μBondapak C_{18}. Temperature of column: ambient (21–25°C). Injection volume: 10 μl of ethyl acetate extract, representing 2 μl of wine, 9.0 nmol of histamine, 7.3 nmol of tyramine and 11.3 nmol of putrescine. Mobile phase: linear gradient elution from 0.02 *M* acetic acid–acetonitrile (90:10) to methanol–acetonitrile (50:50) in 30 min. Flow-rate: 1.5 ml/min. Detector: Waters fluorimeter, Model 420, excitation 360 nm, emission 460 nm, sensitivity × 32, 10 mV full-scale. Peaks: HI = histamine; PU = putrescine; TY = tyramine. Continuous line: chromatogram of derivatized wine. Broken line: chromatogram of derivatized wine spiked with amines.

been reported to be stable for an appreciable length of time after their preparation[10]. This reagent has also been used successfully for the determination of histamine and other amines in wine by Battaglia and Frölich[5,6].

A Villard Noir red wine spiked with different amines and derivatized with Dns-Cl was used to develop the chromatographic procedure. Various mobile phases were tried in order to achieve the best separation of a number of amines from the wine. Satisfactory resolution of tyramine and histamine was achieved in 15 min using a water–methanol gradient. Histamine, tyramine and putrescine were also successfully resolved from the wine extract in 30 min using a gradient of 0.02 *M* acetic acid–methanol–acetonitrile (Fig. 1). Because of the numerous peaks in the chromatograms, simultaneous resolution of more than three amines was not achieved.

Some of these peaks could be from phenol derivatives of Dns-Cl, as red wines contain a high concentration of phenolic compounds and phenols do react with Dns-Cl[11]. The same author also reported that ammonia from the air and in solvents reacts readily with Dns-Cl to form Dns-sulfonamide; in addition, basic amino acids tend to form a small percentage of secondary products during Dns derivatization. Because the separation of a number of amines from a wine sample could not be achieved with Dns-Cl, another reagent was tried.

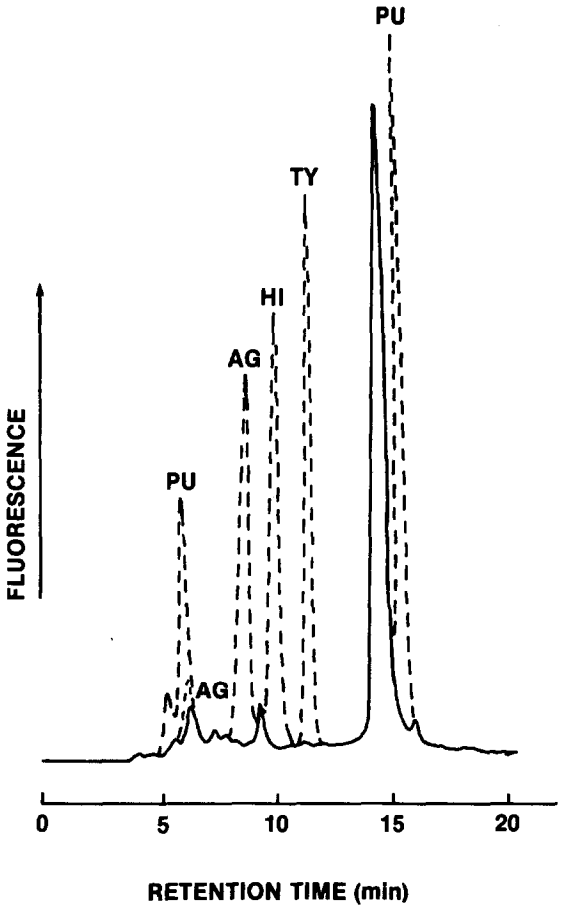

Fig. 2. Reversed-phase HPLC separation of agmatine, histamine, putrescine and tyramine from Villard Noir wine derivatized with fluorescamine. Instrument: Waters Assoc. Column: 30 cm × 3.9 mm I.D., μBondapak C_{18}. Temperature of column: ambient (21–25°C). Injection volume: 20 μl, representing 7 μl of wine and 26 nmol of each amine. Mobile phase: linear gradient elution from 0.08 *M* acetic acid–methanol–ethyl acetate (48:46:6) to 100% methanol in 20 min. Flow-rate: 1 ml/min. Detector: Waters fluorimeter, Model 420, excitation 395 nm interference, emission 460 nm, sensitivity × 4, 10 mV full-scale. Peaks: AG = agmatine; HI = histamine; Pu = putrescine; TY = tyramine. Continuous line: chromatogram of derivatized wine. Broken line: chromatogram of derivatized wine spiked with amines.

Derivatization with fluorescamine

Fluorescamine was tried because of its rapid, almost instantaneous reaction with amino groups at ambient temperature. Numerous isocratic and gradient mobile phases were investigated. The separation of eight amine standards was achieved in less than 30 min. However, di- and polyamines produced more than one peak when reacted with fluorescamine. This could be attributed to the formation of mono- and difluorescamine derivatives[8].

A system was developed to separate agmatine, histamine, putrescine and tyramine in red wine in 20 min (Fig. 2), but another derivatization reagent was considered

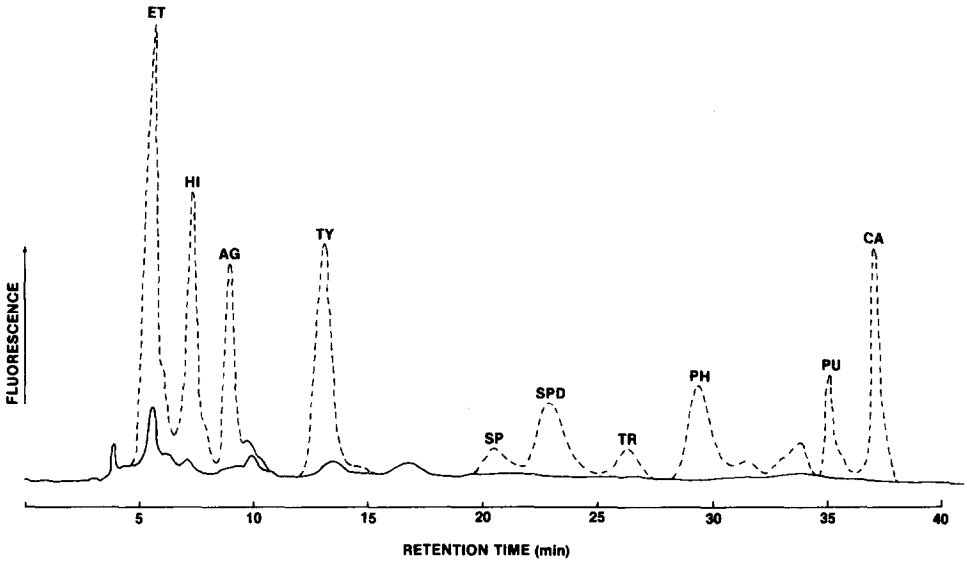

Fig. 3. Reversed-phase HPLC separation of agmatine, cadaverine, ethanolamine, histamine, phenylethylamine, putrescine, tryptamine, tyramine, spermidine and spermine from Villard Noir wine derivatized with OPT. Instrument: Spectra-Physics SP8000B. Column: 30 cm × 3.9 mm I.D., μBondapak C_{18}. Temperature of column: 35°C. Injection volume: 10 μl of ethyl acetate extract, representing 2.5 μl of wine and 125 pmol of each amine. Mobile phase: isocratic mode for 18 min with methanol–0.08 *M* acetic acid–acetonitrile (52:45:3) then linear gradient elution from methanol–0.08 *M* acetic acid–acetonitrile (52:45:3) to 100% methanol in 15 min. Flow-rate: 1 ml/min. Detector: Waters fluorimeter, Model 420, excitation 340 nm, emission 440 nm, sensitivity × 128, 10 mV full-scale. Peaks: ET = ethanolamine; HI = histamine; AG = agmatine; TY = tyramine; SP = spermine; SPD = spermidine; TR = tryptamine; PH = phenylethylamine; PU = putrescine; CA = cadaverine. Continuous line: chromatogram of unspiked wine. Broken line: chromatogram of wine spiked with amines.

because extra peaks for certain amines complicated both the resolution and the quantitation of those amines.

Derivatization with o-phthaldialdehyde

The HPLC method proposed by Subden *et al.*[7] for the determination of histamine in wines and musts and involving pre-column derivatization with OPT was tried. It was soon observed, however, that the OPT–histamine complex was unstable. An investigation using three different methods of derivatization with OPT was carried out in order to seek further evidence of this instability[12]. The method of Davis *et al.*[9] was adopted because it proved to give a stable OPT–histamine complex. All samples were injected within 1 h after their derivatization. The method of Davis *et al.*[9] not only has the advantage of giving a stable OPT–amine complex but it is also selective. Amino acids that have reacted with OPT in the presence of mercaptoethanol are still polar molecules and are, therefore, not extracted with ethyl acetate.

After many unfruitful trials, a satisfactory HPLC procedure was developed for the separation of ten amines as OPT derivatives (Fig. 3).

Although a satisfactory separation of the various amines was achieved, the problem of quantitation remained to be solved. This was attempted by using a com-

TABLE I

QUANTITATION OF OPT-AMINE DERIVATIVES: COEFFICIENTS OF VARIATION FOR EIGHT AMINE STANDARDS AND FOUR REPLICATES (CALIBRATION GRAPHS), USING THE PEAK AREA OR THE PEAK AREA ADJUSTED FOR THE INTERNAL STANDARD (IS) AS THE DEPENDENT VARIABLE

Amine	*Coefficient of variation (%)**							
	Replicate 1		*Replicate 2*		*Replicate 3*		*Replicate 4*	
	Area	*Area adjusted for the IS*	*Area*	*Area adjusted for the IS*	*Area*	*Area adjusted for the IS*	*Area*	*Area adjusted for the IS*
Agmatine	33.79	23.62	16.70	< 22.88	9.88	< 37.30	20.39	11.51
Cadaverine	19.92	13.77	20.11	< 22.88	14.33	< 19.76	35.68	30.17
Ethanolamine	10.58	< 19.66	30.23	< 37.59	28.26	< 40.72	16.18	< 23.26
Histamine	10.37	< 21.08	21.07	< 23.20	20.66	< 48.14	21.70	< 23.03
Phenylethylamine	1.96	< 25.26	3.64	< 11.40	8.05	< 35.15	14.77	< 23.35
Putrescine	18.15	10.06	31.39	29.18	19.17	= 19.11	26.19	21.81
Tyramine	17.92	< 22.02	30.28	< 33.45	17.02	< 44.32	39.85	35.85
Spermidine	27.07	= 27.22	16.80	< 23.05	55.22	< 83.46	37.20	< 44.39

* <, Values for the peak area are less than those for the peak area adjusted for the IS; =, values for the peak area are similar to those for the peak area adjusted for the IS.

bination of the internal and external standards methods. Data obtained from four replicates of six concentrations of amine standards were used to establish calibration graphs for each amine. Tryptamine was used as the IS and was added at a level equivalent to 50 pmol when injected. The calibration graphs were calculated by entering either the peak area or the peak area adjusted for the incorporation of the IS as the dependent variable and the concentration of the amines as the independent variable. To obtain an evaluation of precision and/or acceptability of the two sets of data (y = peak area and y = peak area adjusted for IS), coefficients of variation and coefficients of determination were considered. Of the 32 possible comparisons between peak area and peak area adjusted for the IS, 24 coefficients of variation for peak area were equal to or less than the coefficients of variation for peak area adjusted for the IS (Table I). Coefficients of determination that had values of less than 5% for the quadratic term in the regression equation were considered to have effected an insignificant reduction in the error sums of squares. There were 27 quadratic r^2 values less than 5% for the peak area itself and 21 for the peak area adjusted for the IS (Table II). It was concluded that the use of an IS did not improve the precision of the calibration graphs. Consequently, the external standards method was used to prepare the calibration graphs.

The efficiency of the method of analysis (recovery) was tested for seven amines by comparing the peak areas of nine spiked must and wine samples with the peak areas of the same samples not spiked. Each of the seven standards was added at a level equivalent to 100 pmol when injected. The average efficiencies were 85.6% for agmatine, 94.5% for cadaverine, 70.6% for ethanolamine, 107.0% for histamine, 85.9% for phenylethylamine, 92.7% for putrescine and 98.9% for tyramine.

TABLE II

QUANTITATION OF OPT-AMINE DERIVATIVES: COEFFICIENTS OF DETERMINATION FOR THE LINEAR AND THE QUADRATIC REGRESSION COMPONENTS OF FOUR REPLICATES (CALIBRATION GRAPHS), FOR EIGHT AMINE STANDARDS USING THE PEAK AREA OR THE PEAK AREA ADJUSTED FOR THE INTERNAL STANDARD (IS) AS THE DEPENDENT VARIABLE

Amine	*Regression components*	*Coefficient of determination (%)**							
		Replicate 1		*Replicate 2*		*Replicate 3*		*Replicate 4*	
		Area	*Area adjusted for the IS*	*Area*	*Area adjusted for the IS*	*Area*	*Area adjusted for the IS*	*Area*	*Area adjusted for the IS*
Agmatine	Linear	77.93	93.55	96.87	95.03	98.21	87.37	93.60	98.23
	Quadratic	3.19	1.01	na	na	na	8.65 (a)	4.92	na
Cadaverine	Linear	85.71	96.62	87.21	84.96	95.46	94.32	88.85	92.32
	Quadratic	8.73 (a)	na	1.35	0.68	na	4.67	0.02	1.06
Ethanolamine	Linear	98.26	96.53	90.45	87.68	80.43	77.37	96.14	93.59
	Quadratic	na	na	0.04	0.04	1.39	15.63 (a)	na	5.29
Histamine	Linear	97.52	94.98	92.91	92.90	93.10	78.65	92.73	93.25
	Quadratic	na	5.45 (a)	4.05	2.15	3.18	15.14 (a)	0.20	3.25
Phenylethyl-amine	Linear	99.92	93.38	99.76	98.22	98.93	87.57	98.21	97.17
	Quadratic	na	5.90 (a)	na	na	na	11.19 (a)	na	na
Putrescine	Linear	86.16	98.11	52.30	72.44	85.85	91.26	91.85	95.12
	Quadratic	9.15 (a)	na	7.23 (a)	5.04 (a)	1.04	8.22 (a)	0.29	na
Tyramine	Linear	92.87	92.59	86.66	86.40	96.33	84.60	81.28	86.28
	Quadratic	0.02	5.82 (a)	5.76 (a)	3.75	na	11.53 (a)	0.02	1.80
Spermidine	Linear	89.60	93.50	97.35	95.64	80.11	70.43	91.70	89.24
	Quadratic	1.02	3.14	na	na	11.03 (a)	21.31 (a)	2.20	0.80

* na, Not applicable (100% minus linear r^2 is less than 5%); (a), quadratic r^2 is greater than 5% (r^2 = coefficient of determination).

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