Characterization and Quantification of Phenolic Compounds and Flavonoids Commonly Found in Natural Food Products Using HPLC Analysis: An Analytical Approach for Resolving Merged Peaks

Running title: Analytical Resolution of Merged HPLC Peaks

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SUMMARY
The identification and quantification of phenolic compounds and flavonoids in various natural food products is typically conducted using HPLC analysis. The analysis of such compounds is a particularly complex case since most natural food products contain a large number of different phenolic compounds, many of which possess similar chemical characteristics such as polarity which makes complete separation of all eluents extremely difficult. In this work, a methodology which may be used to quantitatively determine the concentration of two eluents with similar retention times, i.e. which show a merged peak in a mixed solution, is presented and validated in a case study of two pairs of phenolic compounds; (Caffeic and Vanillic Acid) and (Ferulic and P-Coumaric Acid). This analysis technique takes advantage of the different absorbances of the two eluents at various wavelengths and thus may be employed to find the concentration of these eluents using UV-VIS absorbance readings even if they are not separated in the HPLC column. The presented methodology could be applied in the future to the HPLC analysis of food products which possess a vast spectrum of phenolic compounds and flavonoids.

Key words: chromatography, HPLC analysis, UV-VIS absorbance, phenolic compounds, flavonoids
INTRODUCTION

Phenolic compounds and flavonoids are a class of natural products which are commonly found in a variety of food products (1-4) including herbs (5), wine (6-8), beer (9), olive oil (10-13), fruits (14,15) and honey (16-20). Despite being present in relatively small concentrations, these compounds are known to impart a number of beneficial properties to these food products such as anti-microbial, food preservation and anti-oxidant properties (8,17,19,21–27). The amount and type of these compounds found in these products is dependent primarily on variety and location, and in the case of honey, floral sources and, thus, they are also sometimes used as chemical fingerprints to trace the geographic and botanical origins of these food products.

The identification and quantification of phenolic compounds and flavonoids in food products is typically conducted using HPLC analysis with a UV-VIS DAD detector (7,25,28–37). The regular modus operandi involves the isolation and extraction of phenolic compounds from the food product, followed by an HPLC run using a gradient mobile phase consisting of two or more reagents, which are typically a polar organic solvent such as methanol or acetonitrile and a weak acid such as phosphoric or acetic acid (30,31,38). The analytes are then identified and quantified by comparison against standard solutions. While this method is perfectly valid and accurate for certain food products, it may however prove to be insufficient for the analysis of products such as olive oil, wine and honey which possess a considerably large assortment of natural products, most of which are chemically related and have similar polarity. This can make separation of peaks problematic, resulting in some cases in amalgamated peaks which could make it difficult to determine the exact concentration of certain compounds, or indeed, in some situations, even to simply ascertain their presence in food products, particularly if most of the peaks in the spectrum are unidentified. In such scenarios, it is extremely unlikely that an analysis based solely on a single HPLC spectrum is sufficient to obtain a completely accurate and reliable characterization and quantification of these compounds.

In view of this, the objective of this work is to propose a method which can be used to identify and quantify with a high degree of certainty fifteen phenolic compounds which are commonly found in a variety of natural food products ranging from honey and olive oil to fruit juices. The specific aim of this developed is the determination of the concentration of phenolic compounds which have overlapping peaks by taking advantage of their diverse absorbances at different wavelengths. Accurate determination of the individual concentrations of phenolic compounds possessing peaks with identical retention times in a mixture is the ultimate objective.
MATERIALS AND METHODS

Preparation of Standards and Phenolic Mixtures

Standard solutions were prepared for the fifteen phenolic compounds and flavonoids being studied, namely: Kaempferol, Luteolin, Phenylacetic acid (Alfa Aeser, Massachusetts, USA), Apigenin (Rotachrom TLC, Budapest, Hungary), Chrysin, Quercetin, P-Coumaric acid, Naringenin (Sigma Aldrich, Darmstadt, Germany), Ferulic acid, Syringic acid, Vanillic acid, Caffeic acid, Ellagic acid, Gallic acid and Benzoic acid (Acros Organics, Geel, Belgium). The solutions were prepared by dissolving the standards in HPLC grade methanol (Ultragradient Grade) (Carlo Erba, Milan, Italy) to produce stock solutions of 100 mg/L which were then used to prepare 50, 40, 30, 20 and 10 mg/L solutions for the standard plots. In addition, a mixture containing 30 mg/L of each phenolic compound in methanol was also prepared. Two separate mixtures of P-Coumaric Acid and Ferulic Acid with concentrations of 50 mg/L and 50 mg/L, and, 30 mg/L and 70 mg/L, respectively were also prepared. Another similar set of mixtures was also prepared using Vanillic Acid and Caffeic Acid. In order to find the optimum wavelength to use for the HPLC DAD-detector, samples of 100 mg/L of each phenolic compound and flavonoid initially had their absorbance measured using a UV-VIS spectrophotometer (Shimadzu UV-2600) over wavelengths between 180 and 480 nm.

HPLC Analysis

The HPLC analysis of the phenolic compounds and flavonoids was conducted using a Waters 2695 Alliance HPLC system (Waters Inc., Milford, USA), equipped with a DAD UV-VIS detector. The separation was conducted using a Waters Sunfire™ C18 reverse-phase chromatography column, 250 mm length and 4.6 mm width, having a particle size of 5 μm. The phenolic standard solutions and mixtures were injected into the system using an auto-injector. A variety of isocratic and gradient mobile phases were tested at different flow rates and column temperatures in an attempt to achieve a suitable separation of the standards.

The gradient method which was eventually chosen following a series of preliminary studies uses a mixture of acetonitrile (Mobile Phase A) (Honey Well HPLC Grade ≥99.9%, Seelze, Germany) and phosphoric acid (Mobile Phase B) which was prepared by dropwise addition of 85% orthophosphoric acid (Sigma Aldrich, Darmstadt, Germany) to HPLC grade water (Carlo Erba, Milan, Italy) until a pH of 2 was reached. The total runtime of the method was 60 minutes and the concentration gradient was varied as follows: a) Initially 5 % A – 95 % B, b) 15 mins 35 % A – 65 % B, c) 20 mins 35 % A – 65 % B, d) 30 mins 40 % A – 60 % B, e) 35 mins 40 % A – 60 % B, f) 40 mins 50 % A – 50 % B, g) 52 mins 70 % A – 30 % B and h) 60 mins 5 % A – 95 % B. A constant flow rate of 0.5 mL/min and a temperature of 5 °C were used. Following an analysis of the UV-VIS spectra of the individual phenolic
standards, three wavelengths were chosen for analysis for this investigation using the HPLC-DAD detector; 210 nm, 280 nm and 360 nm.

RESULTS AND DISCUSSION

Figures 1 shows the chromatograms for the solution containing all 15 phenolic compounds obtained at wavelengths of 210 nm, 280 nm and 360 nm respectively. The retention times and calibration constants based on area and height of peaks for each phenolic compound for every wavelength are presented in Tables 1 and 2, respectively.

As one can observe from the chromatograms in Figure 1, the gradient method used here separates most phenolic compounds reasonably well with most phenolic compounds showing distinct sharp individual peaks. Moreover, while all phenolic compounds show peaks at a wavelength of 210 nm and 280 nm (except for phenylacetic acid at 280 nm), Luteolin, Ferulic Acid, Caffeic Acid, P-Coumaric Acid, Ellagic Acid, Apigenin, Kaempferol and Quercetin also show peaks at 360 nm. These results are in accordance with those obtained from the initial tests conducted using a UV spectrophotometer in order to determine the choice of wavelengths.

However, as one may observe from Figure 1, there are also two pairs of phenolic compounds which have identical retention times and, hence overlapping peaks; Vanillic and Caffeic Acid at 21.0 min and Ferulic and P-Coumaric Acid at 24.5 min. However, this drawback does not necessarily mean that it is impossible to determine the individual concentrations of these phenolic compounds. As evident from the values shown in Tables 1 and 2, each phenolic compound has a different absorption profile. It is possible to take advantage of this property to determine the concentration of each phenolic compound in the mixture by using the standardization constants of the individual phenolic compounds and the total absorbance of the phenolic mixture at different wavelengths.

The method proposed here operates under the assumption that the total area of the peak at a given wavelength, $A_{\text{Total}}^\lambda$, is equal the sum of the individual areas of the phenolic compounds, $A_{Ph_i}^\lambda$, making up the peak, $Ph_i$ and $Ph_j$, at the same wavelength, $\lambda_i$:

$$A_{\text{Total}}^\lambda = A_{Ph_i}^\lambda + A_{Ph_j}^\lambda$$

This relationship is valid for all wavelengths and thus equation /1/ may be used to generate the following simultaneous equations, in the case for the peaks obtained from two different wavelengths:
These equations can be expanded to incorporate the terms defining the concentrations \( C_{ph_1} \) of the phenolic compounds and the standardization gradient \( k_{ph_1} \) and \( y \)-intercept constants \( b_{ph_1} \), which are related to the area \( A_{ph_1} \), through the following equation:

\[
A_{ph_1}^1 = C_{ph_1} k_{ph_1}^1 + b_{ph_1}^1 \quad /4/
\]

And thus:

\[
A_{ph_1}^1 = C_{ph_1} k_{ph_1}^1 + b_{ph_1}^1 + C_{ph_2} k_{ph_2}^1 + b_{ph_2}^1 \quad /5/
\]

\[
A_{ph_2}^2 = C_{ph_1} k_{ph_1}^2 + b_{ph_1}^2 + C_{ph_2} k_{ph_2}^2 + b_{ph_2}^2 \quad /6/
\]

As one may observe from equations /5/ and /6/, the terms \( C_{ph_1} \) and \( C_{ph_2} \), are common for both equations and thus, since all the other terms are known, one may obtain the values for these concentrations by solving the two simultaneous equations. The final values for \( C_{ph_1} \) and \( C_{ph_2} \), may be expressed as follows:

\[
C_{ph_1} = \frac{A_{Total}^1 \left(A_{Total}^1 - b_{ph_1}^1 - b_{ph_2}^2 \right)}{k_{ph_2}^2 - \left( k_{ph_1}^1 k_{ph_2}^2 \right)} \quad /7/
\]

\[
C_{ph_2} = \frac{A_{Total}^2 \left(A_{Total}^2 - b_{ph_1}^2 - b_{ph_2}^1 \right)}{k_{ph_2}^1 - \left( k_{ph_1}^1 k_{ph_2}^2 \right)} \quad /8/
\]
These equations may be used to calculate the concentrations of P-Coumaric and Ferulic Acid since these two phenolics have very similar retention times and absorb to different extents at all of the three wavelengths used here. In the case of Vanillic and Caffeic Acid, the problem is simpler since while the latter absorbs at all three wavelengths, the former absorbs only in the 210 and 280 nm wavelengths. Therefore, equations /7/ and /8/ may be simplified as follows to calculate the concentrations of these phenolics when considering a wavelength of 210 or 280 nm in conjunction with the 360 nm wavelength, since in the latter case $A_{\text{total}}^{\lambda_1}$ is equal to $A_{ph_1}^{\lambda_2}$:

$$C_{ph_1} = \frac{A_{\text{total}}^{\lambda_2} - b_{ph_1}^{\lambda_2}}{k_{ph_1}^{\lambda_2}}$$

/9/

$$C_{ph_2} = \frac{A_{\text{total}}^{\lambda_1} - \left( k_{ph_1}^{\lambda_1} A_{ph_1}^{\lambda_2} - b_{ph_1}^{\lambda_2} + b_{ph_2}^{\lambda_1} \right) - b_{ph_2}^{\lambda_1}}{k_{ph_2}^{\lambda_1}}$$

/10/

where, $Ph_1$ represents the phenolic which absorbs in both of the wavelengths being evaluated, in this case Caffeic Acid, while $Ph_2$ represents the other, i.e. Vanillic Acid and $\lambda_1$ represents the wavelength at which both phenolics absorb, in this case 210 or 280 nm and $\lambda_2$ represents the wavelength at which one phenolic only absorbs, in this case 360 nm.

In order to validate the effectiveness of this method, equations /7/-/10/ were applied to find the concentrations of two solutions each of mixtures of Vanillic and Caffeic Acid (Mixtures 1 and 2) and Ferulic and P-Coumaric Acid (Mixtures 3 and 4) with known concentrations. The concentrations of these mixtures were calculated using the peak areas from three data sets of wavelengths; 210-280 nm, 210-360 nm and 280-360 nm and the results are presented in Table 3. A comparison between the real and the calculated concentrations of the mixtures is also presented in Figure 2.

(Table 3) (Figure 2)

It is evident from the data in Table 3 and the plot in Figure 2 that the values obtained through the equations are extremely similar to the actual concentrations of the individual phenolic acids making up each of the four mixed solutions. In fact, in the case of the mixtures of P-Coumaric Acid and Ferulic Acid, the calculated values were all within ±0.5 mg/L of the actual values, indicating a high degree of accuracy. On the other hand, in the cases of Vanillic Acid and Caffeic Acid mixtures there are slightly more discrepancies between the points, although overall the average predictions of each combination of wavelengths is still very close to the actual values.
These results confirm the validity of equations /7/-/10/ for calculating the concentrations of HPLC analytes with overlapping peaks based on their varying absorbances at different wavelengths. In theory, such a technique should also be applicable when applied to peak height as well as peak area; however, this is only the case if the phenolic compounds in question possess exactly the same retention times. In the cases presented here the two pairs of phenolic compounds have extremely similar but not exact retention times. This means that while a single large peak is obtained in the mixture, the peak is wider as well as higher and thus while the cumulative peak areas of the individual phenolic compounds conform to the assumption presented in equation /1/, the same cannot be said for the cumulative peak heights, i.e. \( H_{\text{Total}}^H \neq H_{Ph_i}^H + H_{Ph_j}^H \), where \( H \) represents the peak height. In fact, this is evident from the results presented in Table 4 and Figure 3, where calculations corresponding to equations /7/-/10/ but based on peak height are presented. As one may observe, the calculated values obtained through this method consistently underestimate the phenolic concentration by a large extent, hence confirming the inadmissibility of this methodology when applied to Peak Height data.

**Table 4**  **(Figure 3)**

At this point it is important to mention the advantages of using the methodology described here to analyze UV-VIS HPLC results. Although typically it is important to ensure that all the analytes separate completely, this is not always so easily achieved, particularly in the case of natural food products such as honey (also evident from previous works /39-41/) which are known to contain over fifty different types of phenolic compounds and flavonoids. In such cases, finding a gradient method which is capable of achieving complete separation of all constituents is almost impossible, especially since many of these phenolic compounds have extremely similar chemical composition and polarities. By using the methodology presented here one may possibly circumvent this problem, particularly if like in the case described here, the gradient method is capable of completely separating the majority of phenolic compounds, and therefore there is no need to develop another method solely to separate a couple of peaks. Moreover, the equations described in this methodology can also be used to conduct a qualitative analysis in order to determine if any unknown compounds possess overlapping peaks with the current compounds being analyzed. If using the equations to calculate the concentrations of two phenolic compounds over multiple pairs of wavelength combinations results in different calculated values then this is indicative that possibly a third, unknown eluent is present and contributing to the peak area. On the other hand, if all combinations of wavelengths return the same concentrations then this confirms that only the two phenolics in question are present at this retention time. Currently, the standard method used to counteract this problem is to either use multiple UV-VIS absorption-based HPLC protocols with different gradient methods and/or mobile phases such as that employed by
Mradu et al. (35) or else to validate the initial HPLC results using additional detectors such as a mass spectrometer (39,42-44). The methodology proposed in this work eliminates the need of using such techniques as a validation method for a UV-VIS absorption-based HPLC analysis. This would facilitate the analysis of complex solutions since all the results required for this analysis may be obtained from a single HPLC run. However, it should be emphasized that the technique proposed here would replace these techniques for validation and quantification purposes only and that the use of additional methods such as MS-HPLC is still required for the eventual characterization and identification of any unknown natural products within the HPLC spectrum. Furthermore, this technique could also be potentially employed as a quality control method for the analysis of synthetic products containing phenolic compounds and flavonoids. In such cases, where the constituents are already known, a partial HPLC separation coupled with the methodology applied here could be sufficient to quantify the individual phenolic compound content. It is also envisaged that the same concept applied here to find the concentration of phenolic compounds with overlapping peaks should also apply to other eluents and compounds with similar retention times and is not limited solely to the two sets of examples mentioned here.

CONCLUSION
In this work, an HPLC analysis methodology which may be used to find the concentrations of eluents possessing similar retention times within a mixture has been presented and validated. The analysis was conducted on a mixture of fifteen phenolic compounds with two pairs of phenolic compounds possessing peaks with nearly identical retention times using absorbance readings taken using an HPLC-DAD detector. The results obtained from the equations used to calculate the concentrations based on the peak area standardization constants of the individual phenolic compounds showed excellent agreement with the known concentrations of the mixtures and indicate that this technique could be a viable method to quantitatively analyze the concentration of such eluents. It is envisaged that this technique could be applied for HPLC analysis of food products such as olive oil, fruit juices and honey which possess a vast spectrum of phenolic compounds and flavonoids with similar chemical characteristics and thus yield complex chromatograms which are extremely difficult to accurately interpret.

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CONFLICTS OF INTEREST
None.

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Table 1. Retention times, $t_r$, and absorbance constants, $k$, $b$, coefficient of determination, $R^2$, at wavelengths of 210 nm, 280 nm and 360 nm based on the area under the peak obtained through numerical integration.

<table>
<thead>
<tr>
<th>Phenolic Compound</th>
<th>$t_r$(min)</th>
<th>$b_{210}$/L/mg</th>
<th>$R^2_{210}$</th>
<th>$k_{280}$/L/mg</th>
<th>$b_{280}$</th>
<th>$R^2_{280}$</th>
<th>$k_{360}$/L/mg</th>
<th>$b_{360}$</th>
<th>$R^2_{360}$</th>
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Table 2. Retention times, $t_r$, and absorbance constants $k$, $b$, coefficient of determination, $R^2$, at wavelengths of 210 nm, 280 nm and 360 nm based on peak height

<table>
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<tr>
<th>Phenolic Compound</th>
<th>$t_r$/(min)</th>
<th>$k_{210}$/ (L/mg)</th>
<th>$b_{210}$</th>
<th>$R^2_{210}$</th>
<th>$k_{280}$/ (L/mg)</th>
<th>$b_{280}$</th>
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<td>1669</td>
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<tr>
<td>Syringic Acid</td>
<td>20.3</td>
<td>11132</td>
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<td>5124.1</td>
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<td>Quercetin</td>
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<td>4983.7</td>
<td>-2190</td>
<td>0.9475</td>
<td>3075</td>
<td>854.5</td>
<td>0.9699</td>
<td>1065.7</td>
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<td>2980.3</td>
<td>4611.5</td>
<td>0.9979</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
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Table 3. Respective concentrations of the additional mixtures of phenolic compounds with similar retention times which were experimentally measured and calculated using the Peak Area Method

<table>
<thead>
<tr>
<th>Mixture</th>
<th>Wavelengths $\lambda_1$ and $\lambda_2$ /nm</th>
<th>$A_{Total}^{A_1}$</th>
<th>$A_{Total}^{A_2}$</th>
<th>$C_{Caffeic Acid}$ / (mg/L)</th>
<th>$C_{Vanillic Acid}$ / (mg/L)</th>
<th>$C_{Caffeic Acid}$ / (mg/L)</th>
<th>$C_{Vanillic Acid}$ / (mg/L)</th>
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<tbody>
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<td>19094066</td>
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<td>1631556</td>
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<td>50.51</td>
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<td>11247709</td>
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<td>2284079</td>
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<td>71.28</td>
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<td>210 and 280</td>
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<td>15649025</td>
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<td>69.71</td>
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Table 4. Respective concentrations of the additional mixtures of phenolic compounds with similar retention times which were experimentally measured and calculated using the Peak Height Method

<table>
<thead>
<tr>
<th>Mixture</th>
<th>Wavelengths</th>
<th>Real Concentration</th>
<th>Experimental/Calculated Concentration</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$\lambda_1$ and $\lambda_2$ / (nm)</td>
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<td>$H^\lambda_2$ Total</td>
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<td>210 and 360</td>
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<td>280 and 360</td>
<td>700549</td>
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<td>2</td>
<td>210 and 280</td>
<td>1193500</td>
<td>827755</td>
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<td>210 and 360</td>
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<td>280 and 360</td>
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<tr>
<td>3</td>
<td>210 and 280</td>
<td>735240</td>
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<td>210 and 280</td>
<td>740386</td>
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<td>776925</td>
<td>104352</td>
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Fig. 1. Chromatograms showing the peaks obtained for the mixture containing all 15 phenolic compounds (30 mg/L) at 210 nm, 280 nm and 360 nm wavelengths. The peaks represent the following phenolics: 1) Gallic Acid, 2) Syringic Acid, 3) Caffeic Acid, 4) Vanillic Acid, 5) Ellagic Acid, 6) P-Coumaric Acid, 7) Ferulic Acid, 8) Benzoic Acid, 9) Phenylacetic Acid, 10) Luteolin, 11) Quercetin, 12) Apigenin, 13) Naringenin, 14) Kaempferol and 15) Chrysin. Note that not all phenolic compounds show peaks on all of the three wavelengths tested and that Caffeic and Vanillic Acid (3, 4) and P-Coumaric and Ferulic Acid (6, 7) show only one joined peak each at 21.0 and 24.5 minutes respectively.

Fig. 2. Comparison of the actual and calculated concentrations using equations /7/-/10/ presented in Table 3 based on Peak Area for the mixtures of a) Caffeic and Vanillic Acid (Mixtures 1 and 2) and b) Ferulic and P-Coumaric Acid (Mixtures 3 and 4). The straight black line is a guide to indicate the point at which the calculated and actual concentrations are equal.
Fig. 3. Comparison of the actual and calculated concentrations using equations /7/-/10/ based on Peak Height presented in Table 4 for the mixtures of a) Caffeic and Vanillic Acid (Mixtures 1 and 2) and b) Ferulic and P-Coumaric Acid (Mixtures 3 and 4). The straight black line is a guide to indicate the point at which the calculated and actual concentrations are equal.