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Calculation of the molar absorptivity of polyphenols by using liquid chromatography with diode array detection: the case of carnosic acid

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Abstract

Antioxidant activity of vegetable extracts is related to the nature and the amount of active components, mainly polyphenols; therefore, a correct quantification of these molecules should be required to define their concentration in such kind of vegetable extracts. A fast and accurate method to calculate molar absorption coefficients (ε), by using HPLC, has been tested on standard polyphenols and caffeine, and should be widely adapted for standardless quantitative analysis. Molar absorptivity (ε) of carnosic acid (CA) was determined from 200 to 300 nm, by the proposed method and those values were compared to *tert*-butyl-hydroxytoluene (BHT) ones for further comparative quantification.

Keywords: Molar absorptivity; Absolute quantification; Carnosic acid; Polyphenols

1. Introduction

Carnosic acid (CA, Fig. 1) is a phenolic diterpene considered to be the most important antioxidant molecule in sage (*Salvia officinalis*) and rosemary (*Rosmarinus officinalis*) extracts [1–9] and its amount represents a quality parameter for such kind of products. However, it is a quite labile molecule in hydrophilic media, it is shortly stable in the solvents in which it can be isolated or analysed, and its degradation is enhanced by light or high temperatures [2,3,10–12].

CA has also been postulated to be the precursor, in vegetables, of other diterpenes, i.e. carnosol (CAR, Fig. 1) and rosmanol [10,13].

A correct quantification of that molecule should be hoped for, but a pure, commercial standard of carnosic acid is not available and its quantification in vegetable extracts, by UV detection, is generally based only on its chromatographic area, or is achieved by the use of other external standards [3,6–8].

Several authors reported to have isolated CA and other carnosics by extraction methods from dried leaves, or by preparative liquid chromatographic procedures from extracts [3–5,9,10,14–16]. Anyway, there are not published data on their UV response (expressed as absorptivity, or ε) and the quantification of such molecules is delegated to arbitrary choices of each single operator.

Lambert and Beer's law is here applied to relate absorbance (A) with molar concentration (C) of analytes, using the well-known equation: $A = \varepsilon lC$, where the path length through the sample (l) is expressed in cm and ε the molar absorptivity, expressed in $\mathrm{l\,M^{-1}\,cm^{-1}}$.

Molar absorptivity depends on the number and kind of chromophores of the analytes and it is a measure of the electronic absorption, at the wavelength chosen for the detection; further, the solvent used may produce a red shift of absorption (batochromic effect), a blue shift (hypsochromic effect), an increase (hyperchromic effect), or a decrease (hypochromic effect) in absorption intensity [17].

For these reasons, a method to find out an ε value directly from a chromatographic calibration curve could present some difficulties, but it would be particularly useful when not perfectly pure standards are available, for which the ε values, obtained by spectrophotometry, should be affected by a sensible error.

Another purpose of this work is to extend the ε value, obtained at one wavelength for CA, to all the wavelengths of the UV spectrum, and to relate those to the ε values of *tert*-butyl-hydroxytoluene (BHT), which is more stable and

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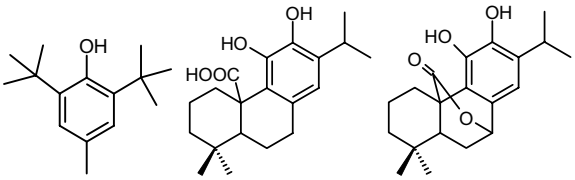

Fig. 1. Structure of *tert*-butyl-hydroxytoluene, carnosic acid and carnosol.

easily available at high purity and low price. Thus, the quantification of CA in vegetable extracts would be definitely free from the demand of its standard.

2. Materials and methods

Pure standard molecules were purchased from Sigma (St. Louis, MO, USA). Minimum purity was 99.9% for caffeine (CAF), 99% for *tert*-butyl-hydroxytoluene, 98% for gallic acid (GA), (−)epigallocatechin (EGC), (+)catechin (C) and (−)gallocatechingallate (GCG), 95% for (−)epigallocatechingallate (EGCG); the purity of (−)epicatechin (EC) was not specified.

Carnosic acid was extracted with ethanol from 567 g of dried leaves of rosemary, by the procedure of Paris et al. [14] modified by Richheimer et al. [9]. Extracting mixture was reduced to small volume and diluted with *n*-hexane; it was after extracted with 5% $NaHCO_3$, treated with H_3PO_4 and reextracted with *n*-hexane. This solution was concentrated and crystals were isolated in *n*-heptane. Extraction solvents and reagents were analytical grade.

Purity of crystallized CA was evaluated by ^{1}H NMR on a AC-200 (200 MHz) Bruker spectrometer (Rheinstetten, Germany), equipped with an ASPECT-3000 workstation and DISR/90-NMR software. NMR spectrum of CA was recorded and compared with published NMR data [4,9]. The height of NMR signal due to the aromatic proton on the C-14 of CA was compared with the ^{1}H signal of the external standard (methanol) and the effective purity of CA was deduced.

CA and BHT were analyzed by HPLC-DAD on a Waters (St. Quentin en Yvelines, France) Alliance 2695 chromatograph, equipped with a PDA 2996 detector, and a Millenium 32 software for data elaboration.

An acetronitrile/0.5% acetic acid in water (15:85; solvent A) to methanol (100; solvent B) gradient [4,6] was applied. The multi-step gradient presented a plateau from 76 to 85 min at 72% of solvent B, that ensured the same conditions for CA (RT = 82.1 min) and BHT (RT = 83.1 min) in terms of eluting solvent at their specific retention time.

A 25 cm × 4.6 mm Hypersil 5 μm column (Shandon, Runcorn, UK) was used with a flow rate of 1 ml min^{-1}. The column temperature was 20 °C.

HPLC separation of catechins was performed on a HP Series 1100 (Hewlett-Packard, Wilmington, DE, USA); it was realized by a methanol/0.4% formic acid in water (25:75; solvent C) to 0.3% formic acid in acetonitrile (100; solvent D) gradient [18,19] at room temperature. The linear gradient elution system was: 100% C for 8 min, to 100% D in 33 min, standing at 100% D for 5 min.

A 25 cm × 4.6 mm *Luna* column (Phenomenex, CA, USA) was used with a flow rate of 1 ml min^{-1}.

Chromatographic solvents were HPLC grade. Both HPLC systems were equipped with an autosampler that allowed to vary injection volumes of the samples from 5 to 20 μl; concentration of pure standards were also varied from 5 to 100 mg l^{-1}.

Molar absorptivity (ε) values of BHT (at 220 and 280 nm) were separately derived from spectrophotometric analysis on a Lambda-9 UV-Vis/NIR instrument (Perkin-Elmer, MA, USA). Absorbance was measured using methanol/0.5% acetic acid in water (80:20), as solvent mixture, to reproduce the HPLC conditions at the CA and BHT retention times.

Molar absorptivity (ε) values of catechins were measured at 270 nm, in 0.3% formic acid in water.

3. Results and discussion

3.1. Calculation of the molar absorptivity (ε), by HPLC with UV detection

The Lambert–Beer's law is valid for an UV detector, which is a spectrophotometer submitted to a dynamic and continuous HPLC flow, operating on chromatographically separated molecules.

Averaged absorbance ($\bar{A}$) of an HPLC separated compound (during the time of elution Δt) is expressed as:

$$\bar{A}\ (\mathrm{AU}) = \frac{10^{-3}\ \mathrm{area\ (mAU\ s)}}{\Delta t\ (\mathrm{min}) \times 60\ (\mathrm{s\ min^{-1}})} \tag{1}$$

and, similarly, the averaged concentration ($\bar{c}$), calculated considering the volume eluted during this Δt is:

$$\bar{c}\ (\mathrm{mol\ l^{-1}}) = \frac{\mathrm{mol}}{\mathrm{volume\ (l)}} = \frac{\mathrm{mol}}{\mathrm{flow\ (ml\ min^{-1})} \cdot \Delta t\ (\mathrm{min}) \times 10^{-3}} \tag{2}$$

It is interesting to underline that the arbitrary choice of Δt (the elution time range of the peak or the complete chromatographic run time) is not influent on the final result; this parameter is present on both the averaged absorbance ($\bar{A}$) and the averaged concentration ($\bar{c}$) and can be simplified.

In fact, the traditional Lambert–Beer's law ($A = \varepsilon lc$) can be easily transformed in:

$$\mathrm{area} = \frac{0.06 \times l \times \varepsilon}{M \times \mathrm{flow}} \times \mathrm{ng} \tag{3}$$

where, if molecular weight (M), path length (l) and chromatographic flow are known, ε results as the slope of the

regression curve built using the injected amounts (ng) and the corresponding areas (mAU s) recorded by the UV detector.

This Eq. (3) can be used either:

1. to calculate the amount of a substance (ng) from its chromatographic area (mAU s), when ε is known;
2. to calculate the ε value from its chromatographic area (mAU s), when the calibration curve is done.

The first application gives a standardless kind of quantification similar to another one described by Torsi et al., defined as an absolute analysis method [20]. It is a strengthening point of the actual treatment to have confirmed, by an alternative argumentation, a result previously achieved by a substantially different way.

The second application is an additional possibility to determine molar absorptivity values (ε), alternative to the traditional spectrophotometric essay. It can be useful for a rapid measure and when the direct spectrophotometric evaluation is affected by an error due to the lack of purity of the standard used.

In order to validate the Eq. (3) for the calculation of the ε values, a traditional spectrophotometric determination was carried out on some commercial pure standards (gallic acid, caffeine, catechins and BHT) and the results were compared to the chromatographic ones (Table 1).

Gallic acid, caffeine and catechins were analysed at 270 nm, in the same solvent mixture previously reported (see Section 2).

BHT was instead analysed at two different wavelengths (220 and 280 nm), in a solvent mixture having the composition of the HPLC gradient at its retention time, not significantly different from that of carnosic acid. Chromatographic dead and dwell volumes would cause a delay of ca. from 1 to 3 min, between the nominal elution time of the chosen gradient and the real solvent mixture passing through the detector cell at that time; in the case of CA and BHT a plateau of 9 min was adequately chosen in the gradient in correspondence to the region of elution of both the molecules so determining quasi-isocratic conditions at their detection time.

The ε values furnished by the two methods were comparable. The catechins' values differ by 1–4% according to the molecules, apart from GA whose the values differ by 12%. The differences could be acceptable as related to the purity of the standards (95% for EGCG and 98% for the other catechins) and to the choice of using the same solvent mixture for gallic acid, caffeine and catechins, not perfectly corresponding to their effective eluent. The ε value of BHT were highly in agreement both at 220 and at 280 nm, differing by only 2 and 1%, respectively.

Table 1
Calculated molar absorptivity (ε) of some phenols and caffeine, by both a traditional spectrophotometric method and a deduction from the HPLC–UV calibration curves

Molecule	λ (nm)	Spectrophotometric method		Chromatographic method	
		ε	S.D.	ε	S.D.
GA[a]	270	10639	117	11955	51
EGC[a]	270	2088	20	2029	45
C[a]	270	2484	40	2588	14
EGCG[a]	270	11920	91	12138	48
EC[a]	270	4290	92	4402	48
GCG[a]	270	11009	53	11131	183
CAF[a]	270	10561	102	10300	149
BHT[b]	220	6887	72	6776	23
BHT[b]	280	1769	18	1754	7

GA: gallic acid; EGC: epigallocatechin; C: catechin; EGCG: epigallocatechingallate; EC: epicatechin; GCG: gallocatechingallate; CAF: caffeine; BHT: *tert*-butyl-hydroxytoluene. Declared purity of standards was kept into account.

[a] Spectrophotometric measures were obtained in 0.3% formic acid in water.

[b] Spectrophotometric measures were obtained in methanol/0.5% acetic acid in water (80:20).

3.2. Application of (3) to the carnosic acid molecule

As previously underlined, the chromatographic method to calculate molar absorptivity values (ε) can be successfully applied to not completely pure samples; it is often the case of species isolated and purified from natural matrixes, such as crystallized CA. One hundred fifteen milligrams of off-white powder, extracted from dried rosemary leaves, gave CA with a purity of 76.8 ± 3.0%, determined by ^{1}H NMR.

The application of (3) for this CA standard ($M = 332$ Da), at 280 nm and an HPLC flow of 1 ml min^{-1} gave: $\varepsilon_{280} = 1131.9 \pm 57.7\,\mathrm{l\,M^{-1}\,cm^{-1}}$. This value has to be referred to the solvent mixture at the carnosic acid retention time (methanol, 0.5% acetic acid in water, acetonitrile, 72:24:4), according to the applied gradient [4,6].

This result was extended to all the wavelengths from 200 to 300 nm, by extrapolation, using the found ε value at 280 nm and knowing the CA UV spectrum profile. The same profile was drawn for *tert*-butyl-hydroxytoluene (BHT), using the experimental value of ε, calculated for the pure molecule, at 280 nm by the same procedure (Fig. 2).

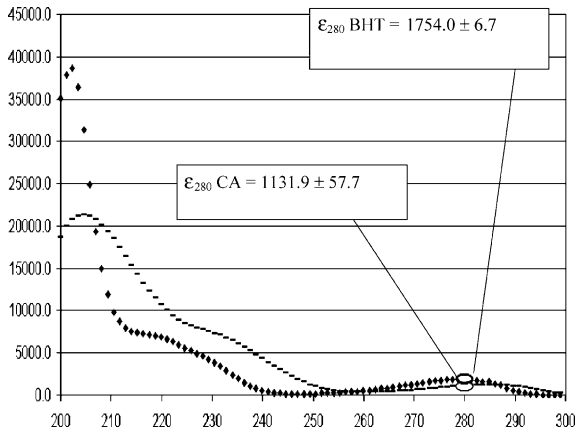

Fig. 2. Graphic representation of BHT and CA ε values, extrapolated from 200 to 300 nm, on the basis of their UV-spectra's profiles.

The ratio between BHT and CA ε values, at 280 nm was:

$$\frac{\varepsilon_{BHT,280}}{\varepsilon_{CA,280}} = 1.550 \pm 0.085$$

that was further confirmed by a producer of vegetable extracts (unpublished results), that using pure CA, linked its spectrophotometric response to that of BHT, in methanol/buffer pH 3 (85:15), at 280 nm, giving:

$$\frac{\varepsilon_{BHT,280}}{\varepsilon_{CA,280}} = 1.573$$

A similar procedure could be applied to carnosol's UV spectrum, for which the relative response towards carnosic acid, at 285 nm, was suggested by the same producer (unpublished results) to be:

$$\frac{\varepsilon_{CAR,285}}{\varepsilon_{CA,285}} = 0.734$$

and the extrapolation could be done from 200 to 300 nm. Such a result for CAR should be considered as a mere indication and should be confirmed by means of selective recovery from the natural source (as sage or rosemary leaves).

Table 2
Molar absorptivity (ε) calculated for *tert*-butyl-hydroxytoluene (BHT) and carnosic acid (CA) from 200 to 300 nm as described in the text

Wavelength (nm)	BHT	CA	Wavelength (nm)	BHT	CA
199.9	34743.7	18750.6	250.5	175.4	1023.5
201.1	37556.6	20160.1	251.7	197.4	829.3
202.3	38280.5	20929.1	252.9	225.2	671.9
203.5	36133.2	21361.1	254.0	258.3	548.3
204.6	31077.1	21453.7	255.2	298.3	456.5
205.8	24728.7	21276.4	256.4	341.8	392.3
207.0	19091.1	20866.7	257.6	390.1	350.7
208.1	14793.4	20252.7	258.8	449.9	351.6
209.3	11742.2	19477.1	259.9	513.0	340.9
210.5	9766.4	18610.7	261.1	583.4	343.9
211.7	8594.7	17617.3	262.3	661.6	357.0
212.8	7906.5	16535.9	263.5	748.4	380.4
214.0	7513.3	15415.5	264.7	840.4	409.8
215.2	7295.3	14337.6	265.9	934.8	445.8
216.4	7172.9	13342.2	267.0	1036.2	487.1
217.5	7088.9	12419.7	268.2	1139.6	533.4
218.7	6989.6	11557.6	269.4	1245.3	585.5
219.9	6826.8	10773.9	270.6	1349.3	643.4
221.1	6573.3	10079.6	271.8	1453.7	705.2
222.2	6248.0	9474.6	273.0	1558.5	770.5
223.4	5886.2	8968.6	274.1	1654.6	837.8
224.6	5531.7	8571.5	275.3	1734.7	905.2
225.8	5203.3	8275.2	276.5	1787.0	970.7
226.9	4885.5	8038.6	277.7	1804.6	1031.4
228.1	4549.0	7825.7	278.9	1791.1	1085.3
229.3	4179.4	7613.5	280.1	**1754.0**	**1131.9**
230.5	3777.6	7386.0	281.2	1707.6	1170.3
231.6	3352.3	7133.7	282.4	1663.8	1202.3
232.8	2894.2	6846.5	283.6	1613.2	1225.4
234.0	2405.0	6513.0	284.8	1523.5	1238.1
235.2	1905.1	6137.9	286.0	1362.3	1235.0
236.4	1441.1	5729.4	287.2	1120.9	1211.9
237.5	1050.9	5295.0	288.4	845.2	1164.7
238.7	743.6	4839.0	289.5	587.5	1093.6
239.9	516.0	4368.3	290.7	385.3	1000.1
241.1	360.0	3897.4	291.9	242.6	889.8
242.2	258.2	3444.6	293.1	150.3	766.9
243.4	197.8	3018.1	294.3	91.4	640.8
244.6	163.8	2614.4	295.5	57.1	519.8
245.8	146.3	2229.1	296.7	37.7	408.6
247.0	141.5	1867.1	297.9	25.7	312.0
248.1	146.1	1540.7	299.0	19.8	231.7
249.3	158.7	1259.2	300.2	13.9	168.2

Experimental values are in bold.

Molar absorptivity values are reported in Table 2 for BHT and CA at all the wavelengths from 200 to 300 nm. On one hand, CA ε values should allow to quantify CA by the Eq. (3) with detection at any wavelengths of HPLC analysis. On the other hand, CA could be also quantified on the basis of BHT calibration curve, by applying the ratio derived from their different ε values here tabulated.

The choice of the latter comparative quantification method could be a way to avoid errors inherent to absolute methods.

Actually, differences from nominal and real instrumental parameters, as the path length and loop injection size, could introduce systematic discrepancy from the exact spectrophotometric values.

A possible way to take into account this kind of errors consists in a preliminary calibration of each single instrument, by a pure standard of well-known absorptivity; constant value appearing in the Eq. (3) would be adequately corrected by really injected amounts (depending on the exact injection volume) and real path length.

Torsi et al. [21] describe, in their experimentation on different HPLC models, systematic errors of 18–20% on some type of instruments.

4. Conclusions

In an HPLC analysis, every detected species should be quantified through its specific response factor, proportional to the molar absorptivity (ε); generally this is obtained by constructing a calibration curve. Nevertheless, many natural molecules cannot be quantified in this way, for the lack of a pure standard, as in the case of carnosic acid.

In this work, the molar absorptivity (ε) of CA, in $\mathrm{l\,M^{-1}\,cm^{-1}}$ at 280 nm, was indirectly obtained by an equation adapting the Lambert–Beer's law to an HPLC–UV detector. ε values were calculated at all the wavelengths from 200 to 300 nm, by an extrapolation on the basis of CA UV spectrum. These values were compared to BHT's ones.

Quantification of CA in vegetable extracts has now to be considered independent from the availability of its pure standards. Absolute quantification by the knowledge of CA's ε values is a suitable technique.

Anyway, comparison with BHT response values would provide an additional tool for quantitative analysis. BHT could be used as internal standard (especially when recoveries should be carefully taken into account) and quantification

of CA could be carried out on the basis of its relative response factors versus BHT.

It is necessary to underline that considerations about comparative quantification of CA descend from the possibility to dispose *una tantum* of its standard of known purity; absorptivity represents an universal chemical parameter from which any kind of comparison should be later allowed.

At this step, the standardless quantification should be applied as a direct and immediate analytical tool (and the knowledge of ε values is required for it) while the comparative analysis through the use of relative response factors could be adapted to a more accurate quantification able to exclude some systematic errors typical of any absolute method.

Quantification of other carnosic derivatives, detected in complex mixtures as vegetable extracts, should be also calculated using their specific ε value, determined first on a standard of known purity. But it is evident that referring their quantification to the carnosic acid spectrophotometric response could be a provisionally acceptable solution, theoretically more correct than providing quantitative results based on crude per cent areas or on extraneous standards.

The elaborated Eq. (3) could be moreover an useful tool to achieve molar absorptivity of other molecules, directly from HPLC data, resulting a valid alternative to the classical spectrophotometric measurement, when the amount of the standards, or their purity are very low.

The ε values derived from this method are referred to the solvent mixture corresponding to the applied HPLC gradient, at the specific retention time of each molecule; acidity of the medium must be taken under control, particularly for weak acids and bases, to avoid dissociation equilibria that could invalidate the correctness of the measurements.

A deep knowledge of instrumental parameters as the exact path length and injection volumes, the dwell volume of the chromatograph, that can affect the solvent conditions at any given retention time, should be considered as precious devices to minimize systematic errors.

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