北京林业大学
Beijing Forestry University

Rapid Detection of Walnut oil by FTIR and Bioactivities of Walnut Protein Hydrolysates

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北京林业大学
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Walnut distribution in China and the World:

	Area (Acre)	Production (Ton)
World	**3.0 m**	**1.7 m**
China	**1.7 m**	**1.0 m**

Walnut distribution in China:

昵图网 www.nipic.com

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- **In walnut:**
  - **oil: 60-70%**
  - **protein: ~20%**

- **Walnut oil processing machine**

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1 **Authentication and adulteration of walnut oil**

2 **Utilization of remained walnut protein**

1 Authentication and adulteration of walnut oil

- **Authentication of walnut oil by GC (Gas Chromatography)**

- **Authentication of walnut oil by FTIR-PCA (Fourier Transform Infrared Spectroscopy combined with Principal Component Analysis)**

- **Adulteration of walnut oil by FTIR-PLS (Partial Least Square)**

1.1 Authentication of walnut oil detected by GC

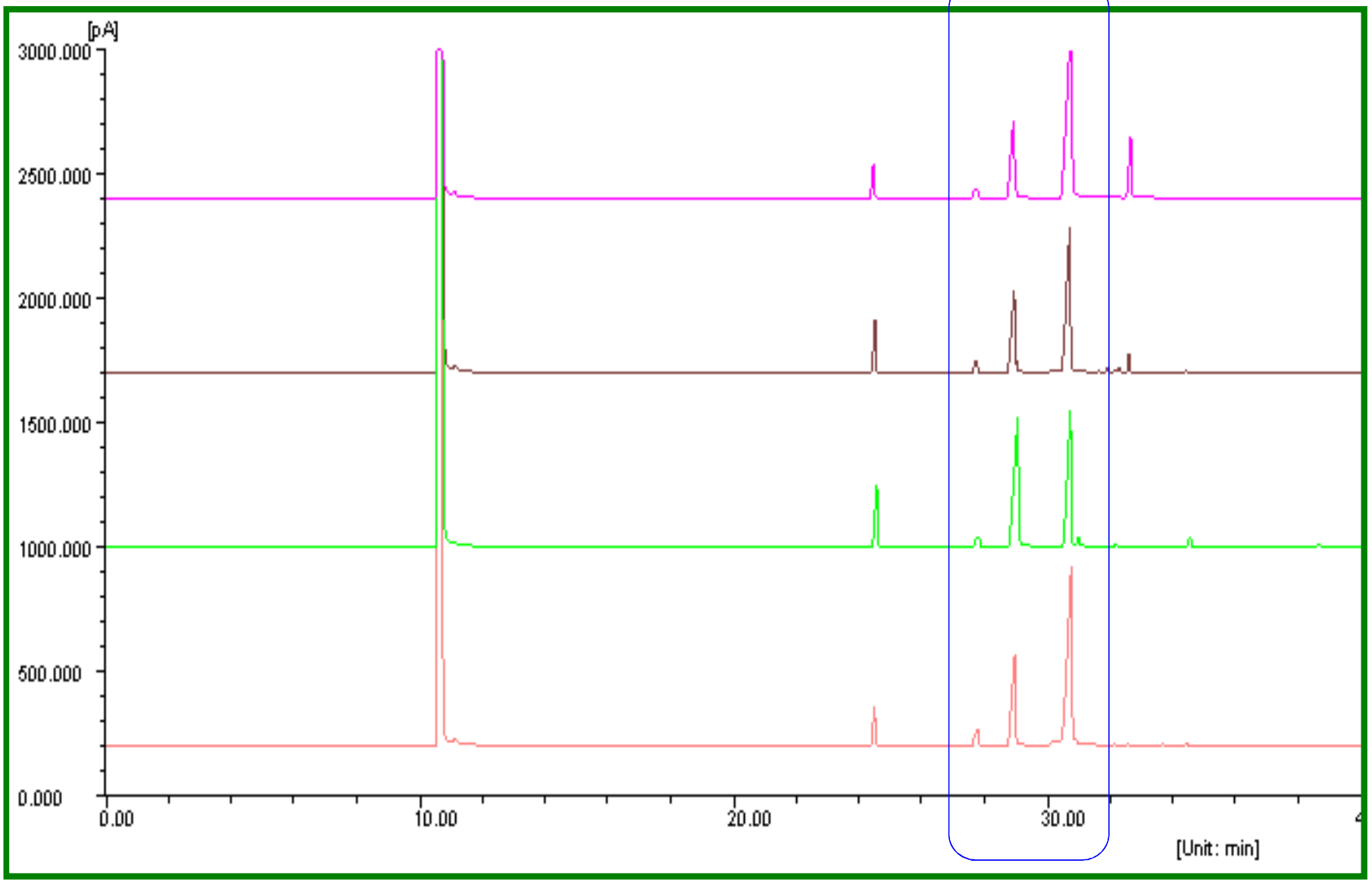
Fatty acids	Walnut oil	Olive oil	Soybean oil	Sunflowerseed oil	Peanut oil
C16:0	6.0~10.0	7.5~20.0	8.0~13.5	5.0~7.6	6.0~10.0
C16:1	0.1~0.5	0.3~3.5	≤0.2	≤0.3	≤1.0
C18:0	2.0~6.0	0.5~5.0	2.5~5.4	2.7~6.5	6.0~10.0
C18:1	11.5~25.0	55.0~83.0	17.7~28.0	14.0~39.4	6.0~10.0
C18:2	50.0~69.0	3.5~21.0	49.8~59	48.3~74.0	6.0~10.0
C18:3	6.5~18.0	≤1.0	5.0~11.0	≤0.3	≤1.0

(From Chinese Standards on edible oils)

Fatty acids	Walnut oil Standard	Sample 1	Sample 2	Sample 3	Sample 4
$C_{16:0}$	6.0-10.0	5.1081	5.1600	5.9068	4.5924
$C_{18:0}$	2.0-6.0	2.4268	2.2855	2.4940	1.7695
$C_{18:1}$	11.5-25.0	18.9071	16.2486	19.3906	18.0029
$C_{18:2}$	50.0-69.0	63.3296	65.3931	60.9328	64.7526
$C_{18:3}$	6.5-18.0	9.7489	8.9847	6.5544	9.7340

◆**Walnut oil has high content of linoleic acid (60-65%) and linolenic acid (6.5-10%), higher than those in other oils.**

◆**Ratio of linoleic acid to oleic acid in walnut oil is between 3.1 and 4.0.**



GC of qualified walnut oils based on fatty acids

★ Comparison of GC and FTIR

- **GC analysis**
  - **Classic method**
  - **Clear and easy to be understood**
  - **Requiring derivatization**
  - **Time-consuming**
  - **Trained techinician**

- **FTIR analysis**
  - **Relatively new analysis method**
  - **Combined with chemometrics**
  - **Little sample preparation**
  - **Minimizing hazard solvent**
  - **Simple experimental procedure**
  - **Rapid**
  - **Cost saving**

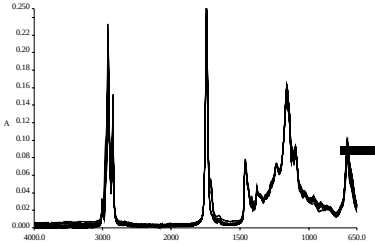
1.2 Qualitative detection on authenticity of walnut oil by FTIR-PCA

Materials： 7 walnut oil, 5 soybean oil, 5 peanut oil, 5 canola oil, 5 blended oil, 5 sunflower oil

FTIR： Nicolet iS5 FTIR

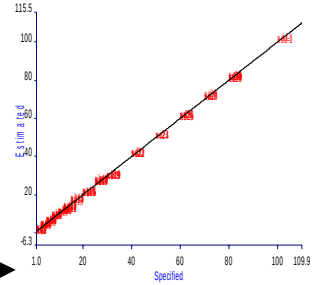
FTIR analysis procedure:

Model samples

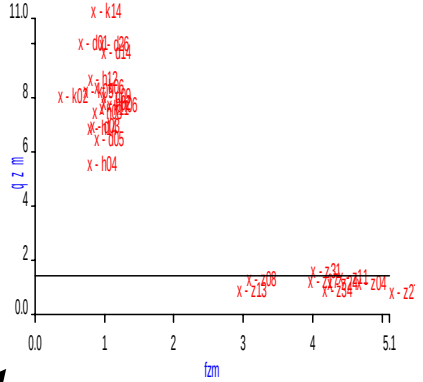


FTIR spectrum

Reference Analysis

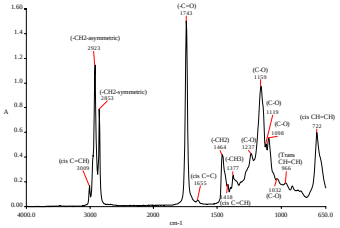


Modeling



Discrimination

Test samples

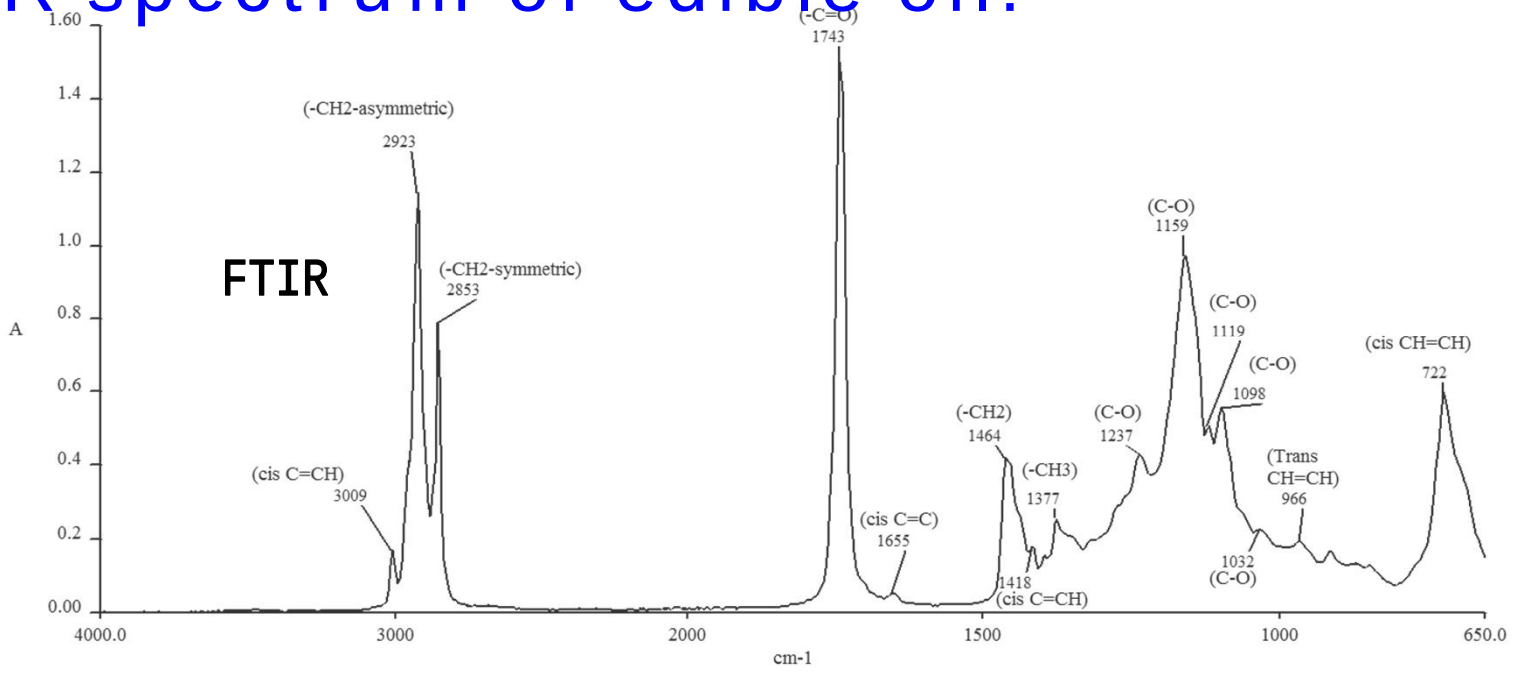


FTIR spectrum

Model prediction

Quantification

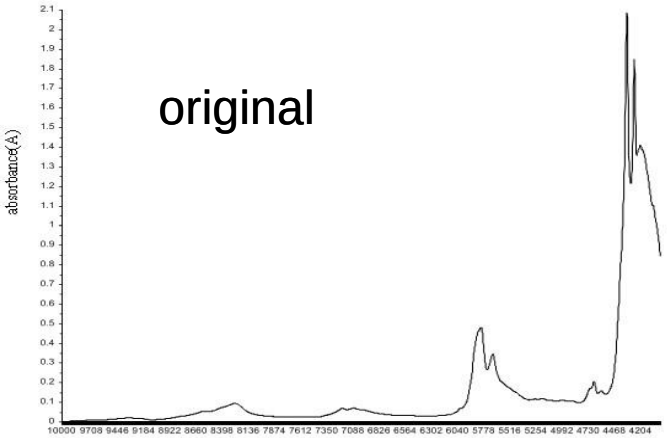
FTIR spectrum of edible oil:



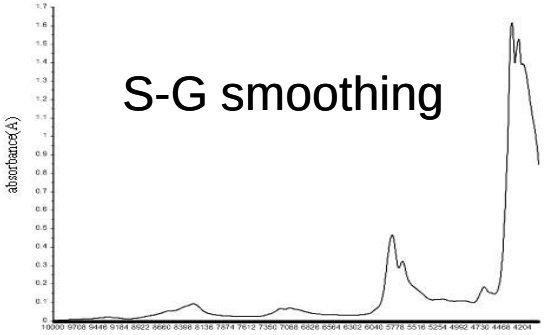
Band (cm^{-1})	Vibration mode	Main attribution	Band (cm^{-1})	Vibration mode	Main attribution
3009	ν (C=C)	unsaturated fatty acid	1237		
2923	ν_{as}(C–H)	methylene	1159		
2853	ν_{s}(C–H)	methylene	1119	ν_{s}(C–O)	triacylglyceride
1743	ν(C=O)	triacylglyceride	1098		
1655	δ(C=C)	unsaturated fatty acid	1032		
1464	δ(C–H)	methylene	966	δ(–HC=CH–)	trans fatty acid
1418	δ(C=C)	unsaturated fatty acid	722	ρ(–HC=CH–)	unsaturated fatty acid
1377	δ_{s}(C–H)	methyl			

Spectrum pretreatment:

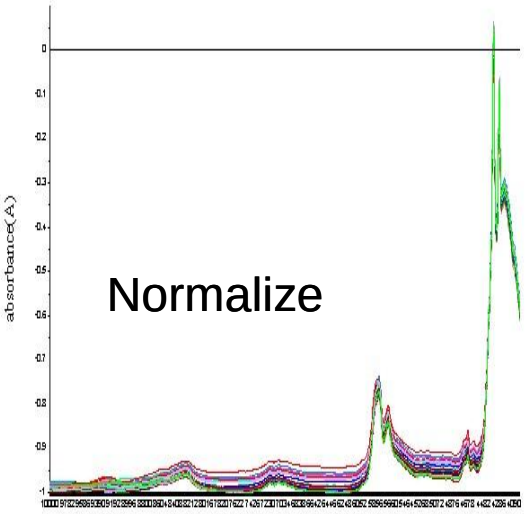
original



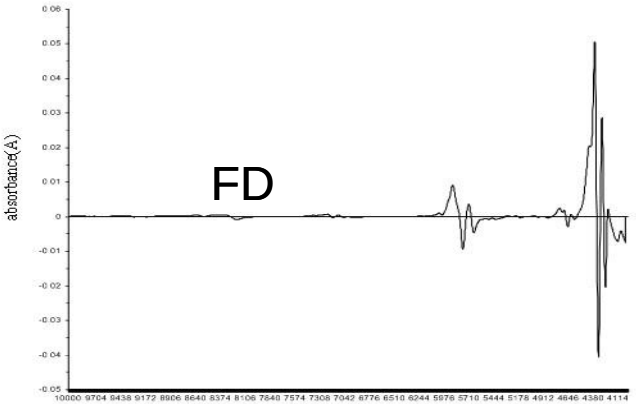
S-G smoothing



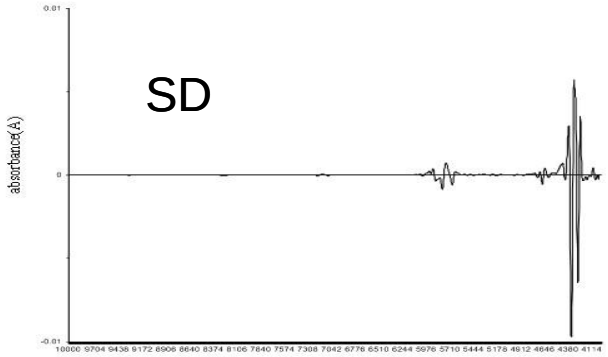
Normalize



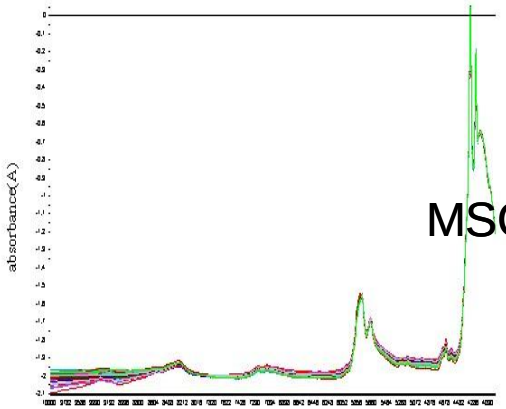
FD



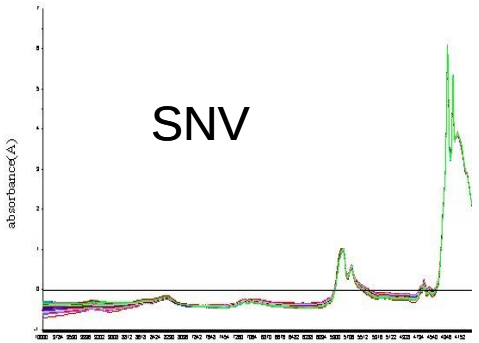
SD



MSC



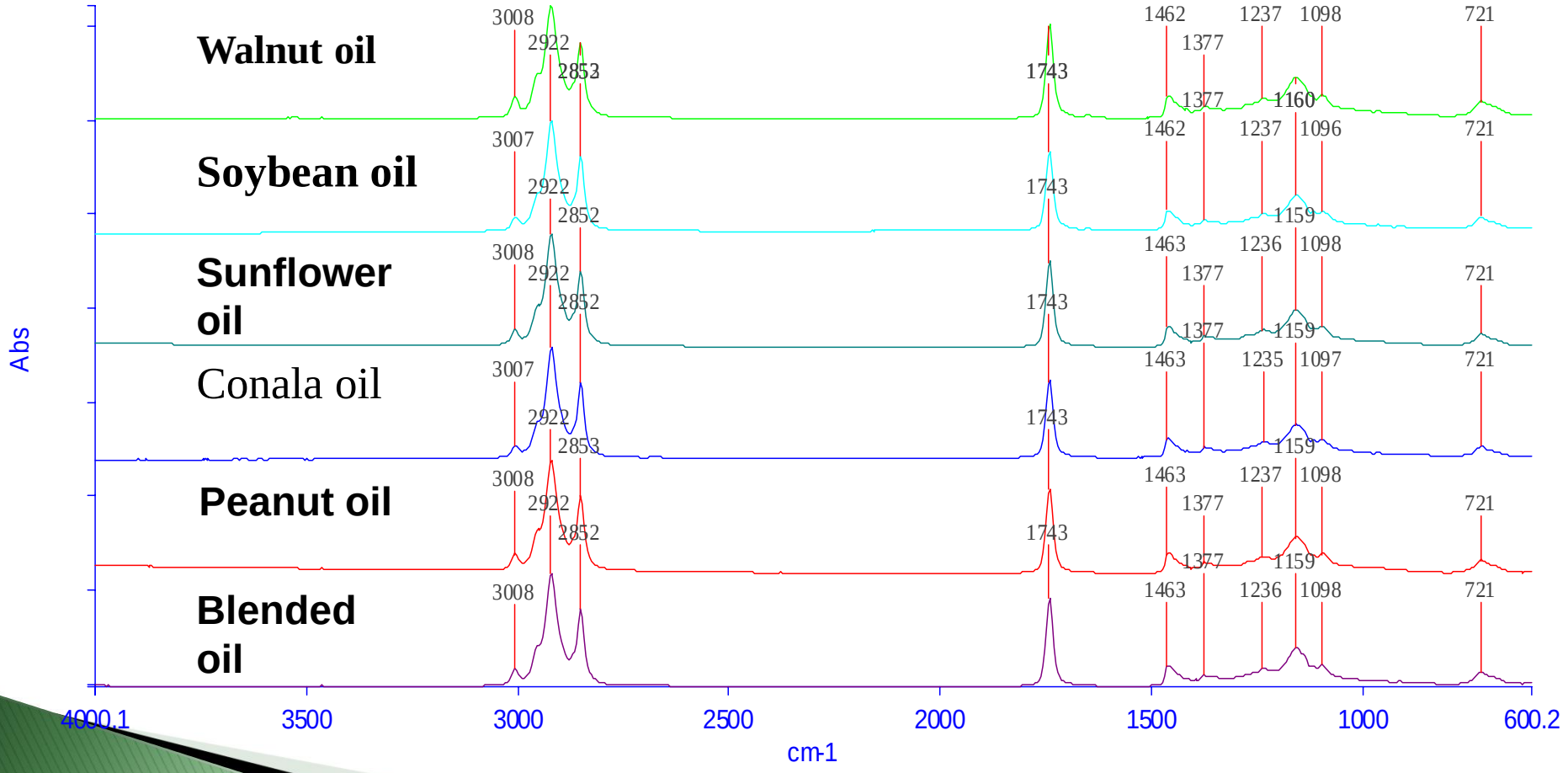
SNV



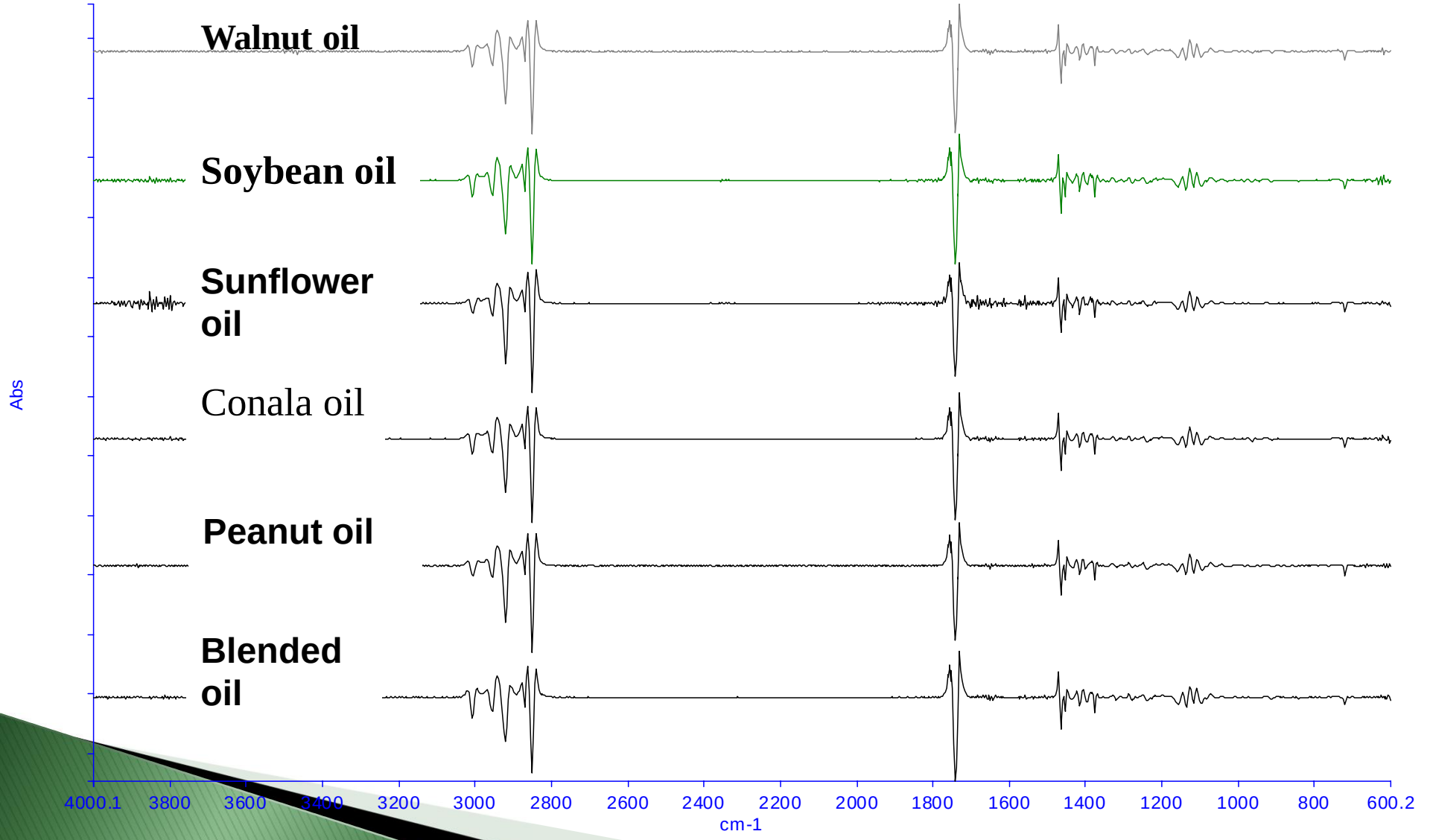
Chemometrics:

- **PCA:Principle Component Analysis**
- **PLS: Partial Least Square**
- **MLR:Multivarate Linear Regression**
- **PCR:Principle Component Regression**
- **ANN: Artificial Neural Net**

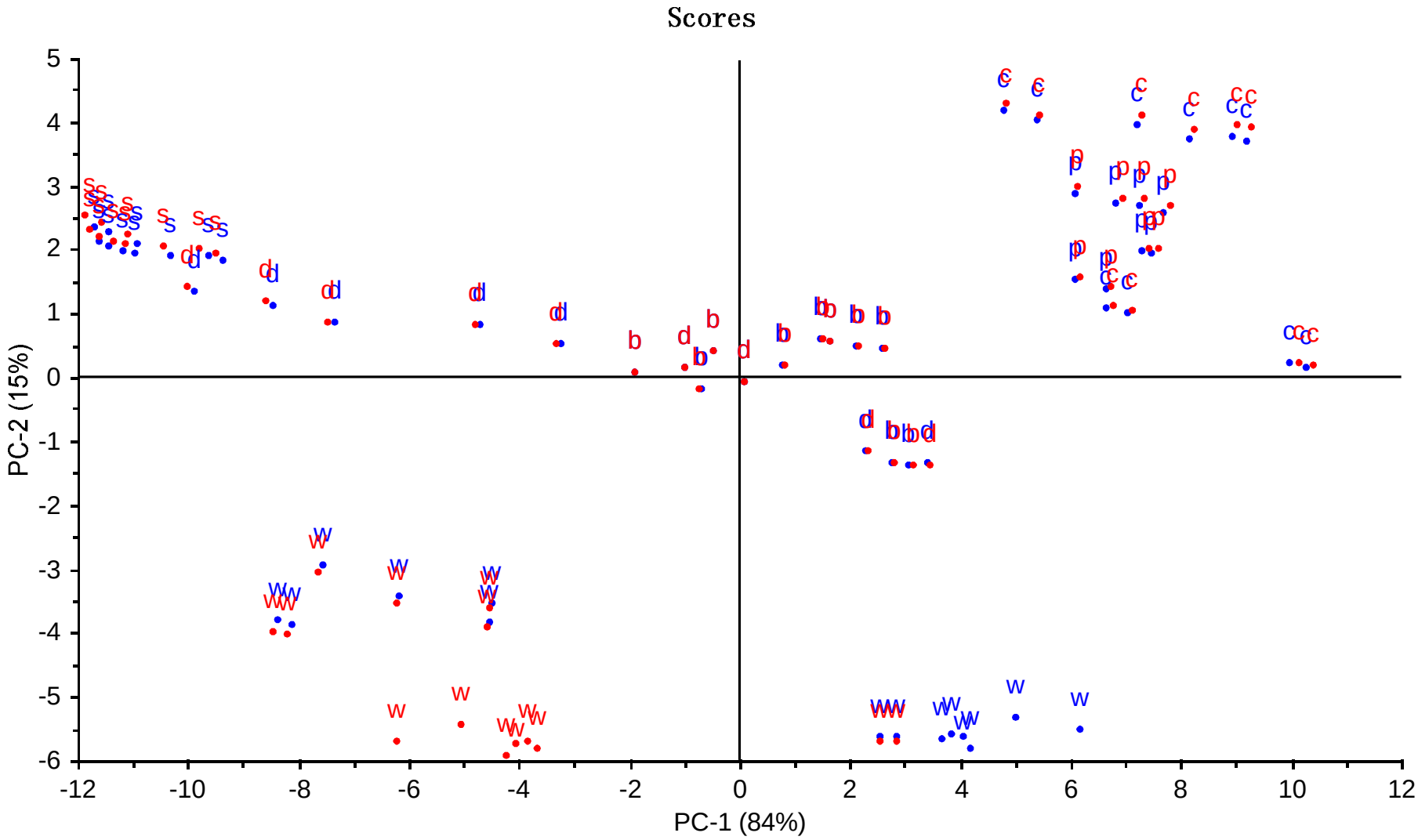
FTIR spectra of walnut oil and other oils:



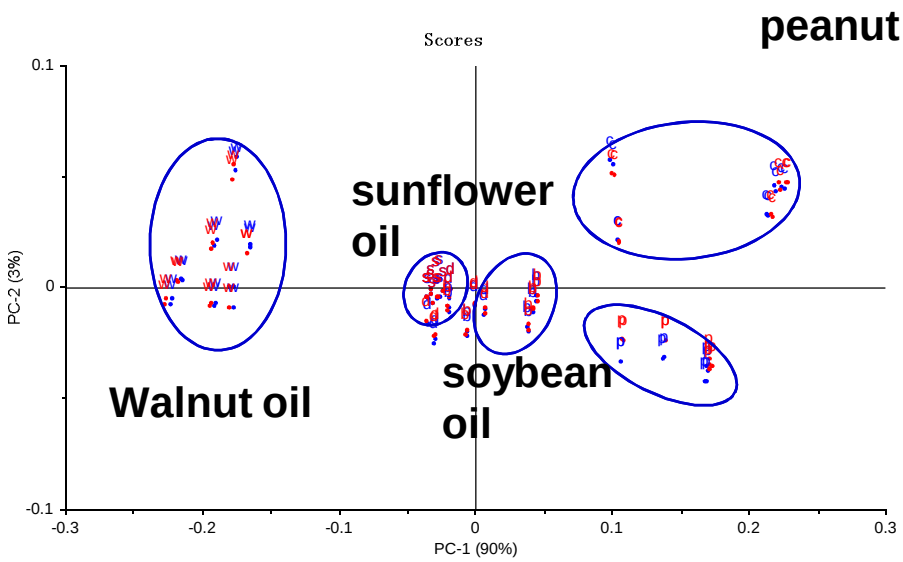
The second derivative of FTIR spectra of walnut oil and other oils:

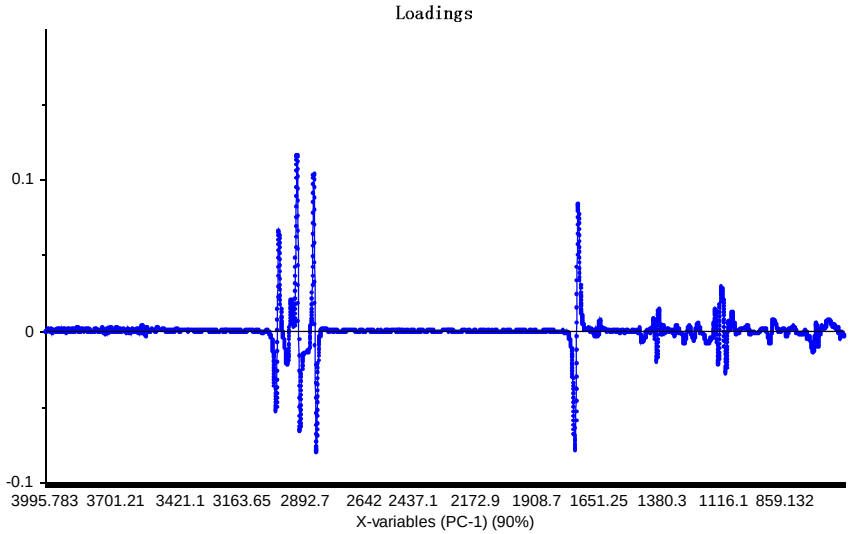


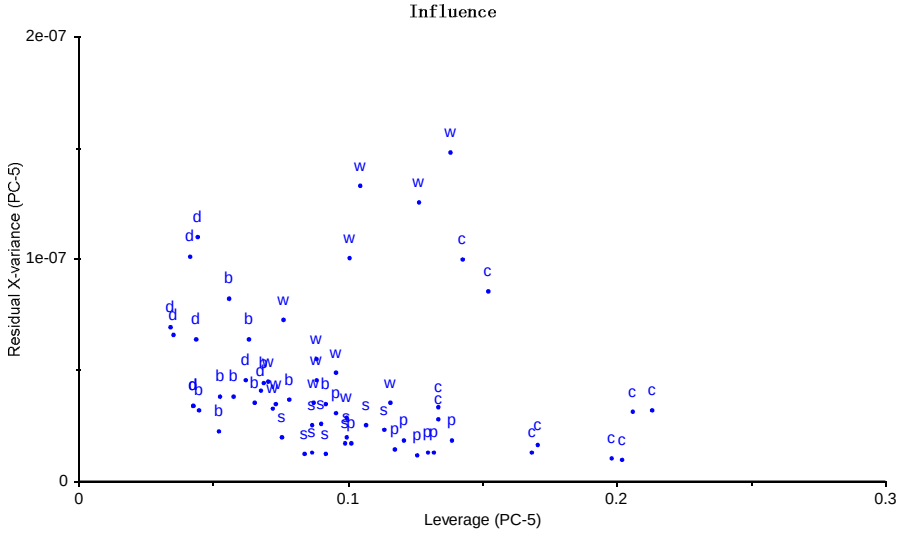
The PCA graph of walnut oil and other oils:

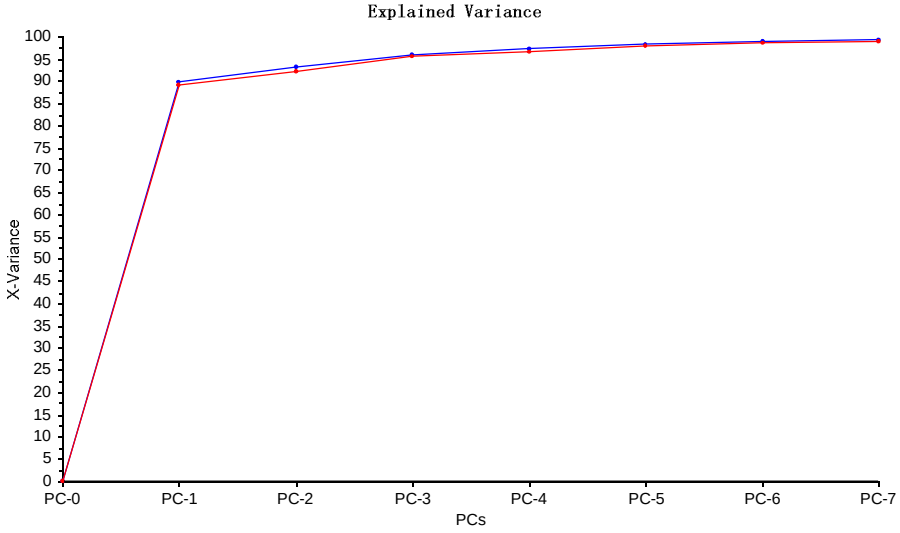


PCA Classification model of the walnut and other oils with SNV and the first derivative:









Conclusion:

- After the first derivative spectrum treatment, walnut oil had no overlap with other oils and they didn’t disturb each other.
- The FTIR-PCA classification model of walnut oil and other oils was practical.

1.3 Quantitative detection on adulteration of walnut oil by FTIR-PCA

- Walnut oil adulterated with soybean oil: 5%、10%、15%、20%、30%、40%、50% adulteration ratio respectively
- Walnut oil adulterated with peanut oil: 5%、10%、15%、20%、30%、40%、50% adulteration ratio respectively
- Totally 32 samples were prepared and used as calibration set，among them 10 samples were used as validation set.

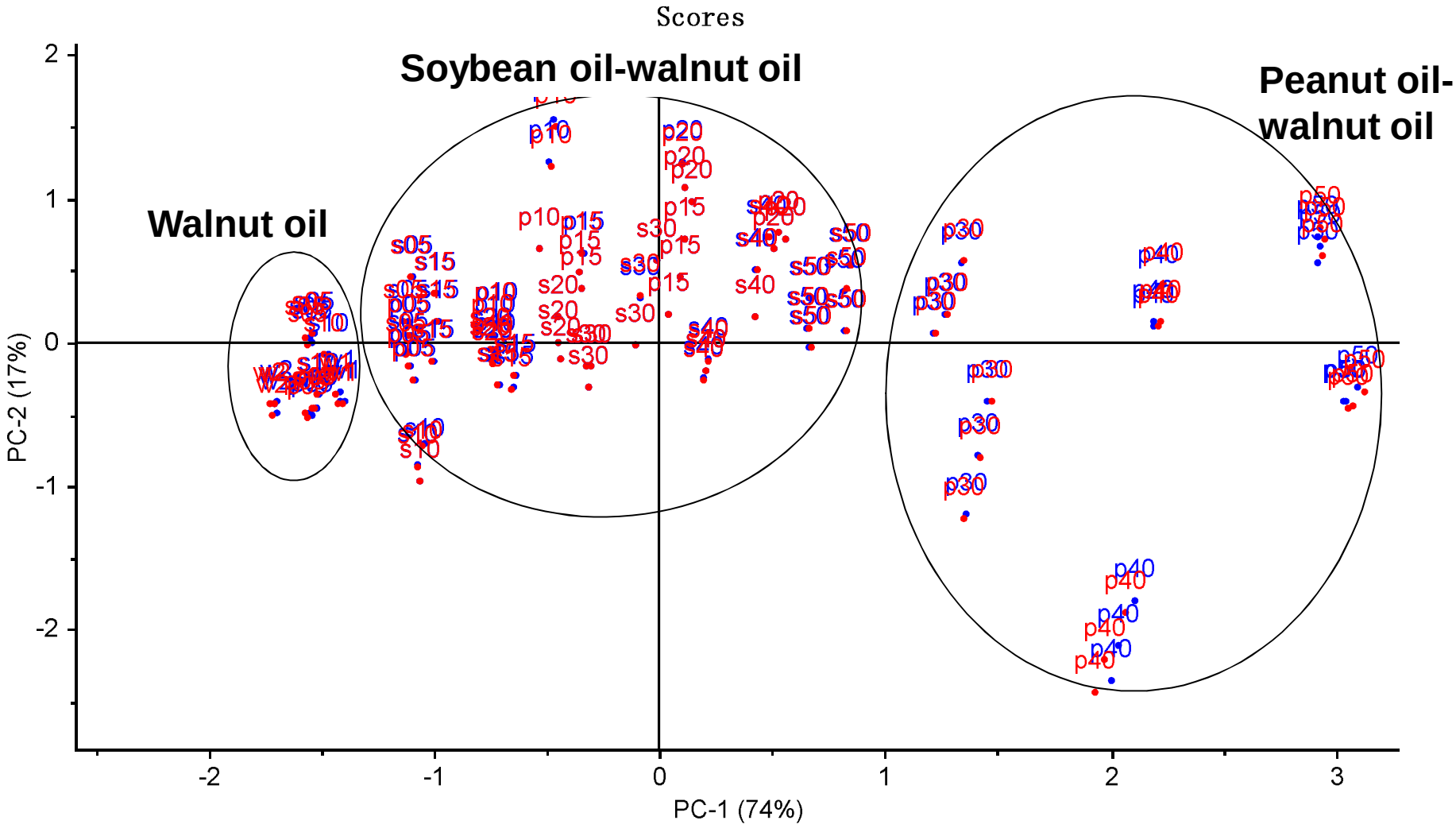


Fig. PCA classification of walnut oil and adulterated walnut oil

Conclusion：

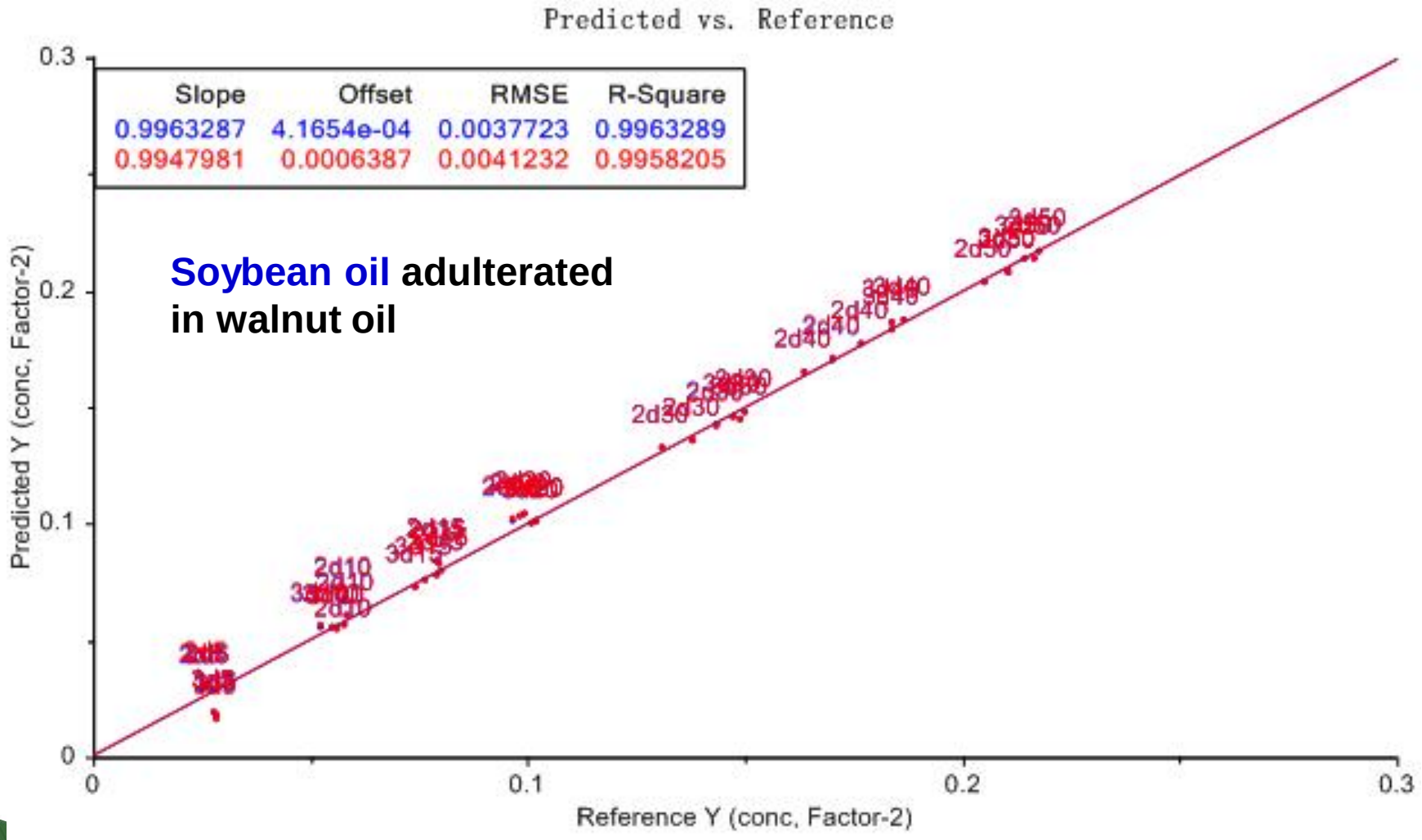
- The determination limits of soybean oil adulterated in walnut oil by PCA was 5%;
- The determination limits of peanut oil adulterated in walnut oil by PCA was 10% .

1.4 Quantitative detection on adulteration of walnut oil by FTIR-PLS

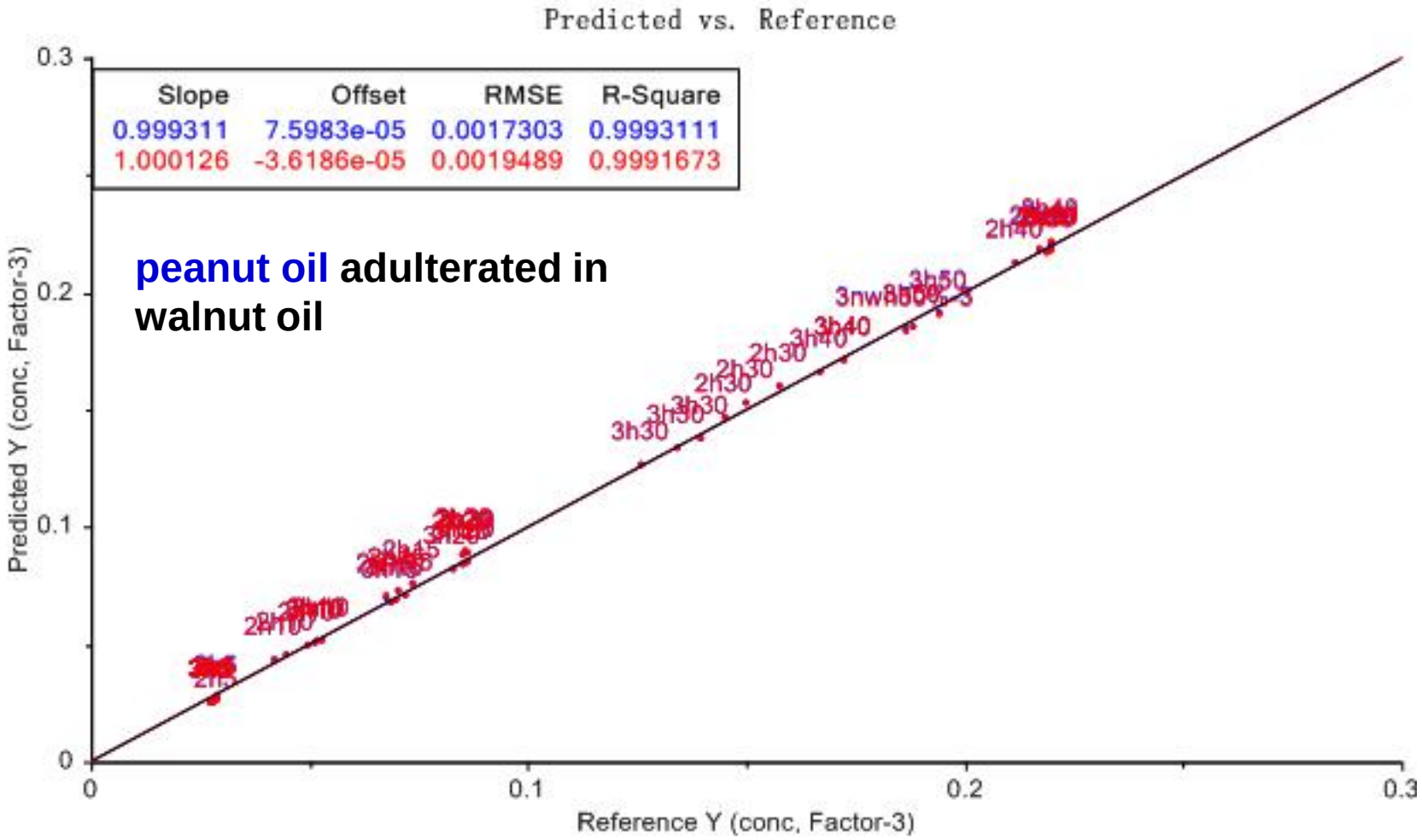
Table Three pretreatment methods for FTIR spectra

Pretreatment	oils	principal components	Calibration		Validation	
			R^2	RMSEC	R^2	RMSECV
Original	Soybean oil	4	0.9928	1.2894	0.9899	1.5271
	Peanut oil	2	0.9886	1.6232	0.9869	1.7017
SNV	Soybean oil	5	0.9984	0.3388	0.9944	0.6781
	Peanut oil	4	0.9881	0.9448	0.9801	1.2297
Normalization	Soybean oil	2	0.9963	0.0038	0.9956	0.0041
	Peanut oil	2	0.9993	0.0017	0.9992	0.0019

After normalization, RMSEC of calibration and RMSECV of validation were decreased and R^2 reached 1.0.



PLS regression calibration curve



PLS regression calibration curve

Predicted results of adulterant amount of walnut oil with soybean oil and peanut oil

Adulterant amount	Soybean oil predicted value (%)	Deviation	Adulterant amount	peanut oil predicted value (%)	Deviation
15.0000	17.0402	2.2824	15.0000	14.8458	2.1988
20.0000	20.8656	2.1129	20.0000	20.6986	1.6011
30.0000	29.2881	1.9541	30.0000	30.8229	1.5331
40.0000	39.8696	1.7609	40.0000	40.1978	1.4868
50.0000	48.8511	1.3909	50.0000	49.6574	1.6690
10.0000	6.8611	1.4271	15.0000	13.4151	1.4393
15.0000	17.4057	1.3087	20.0000	19.2766	1.5082
20.0000	21.7324	1.2299	30.0000	29.1378	1.3886
40.0000	39.1579	1.3292	40.0000	39.2908	1.3089
50.0000	48.3955	1.3749	50.0000	47.8247	1.7312

Conclusion:

- Partial least square (PLS) analysis was used to establish a binary system of adulteration content of walnut oil. Soybean oil and peanut oil volume fraction by PLS calibration model with R^2 and RMSEC value were 0.9963, 0.9993 and 0.0038, 0.0017 respectively.

- Deviation between predicted and reference value was 1.2299～2.2824, which is insignificant and means the calibration model is practical.

2. Bioactivities of walnut protein and hydrolysates

2.1 Separation and purification of angiotensin Ⅰ-converting enzyme inhibitor from walnut protein

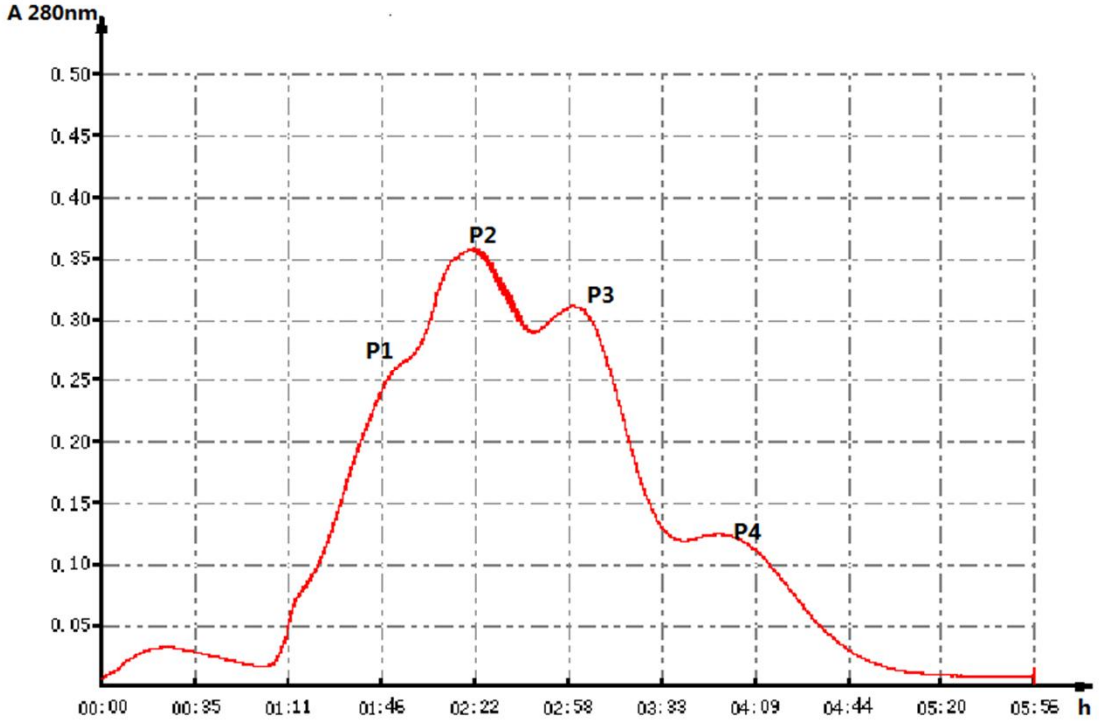
2.2 promotion of spleen lymphocyte and macrophage functions induced by neutral protease hydrolysate of walnut protein

2.3 Inhibition of proliferation induced by papain hydrolysate of walnut protein on tumor cells

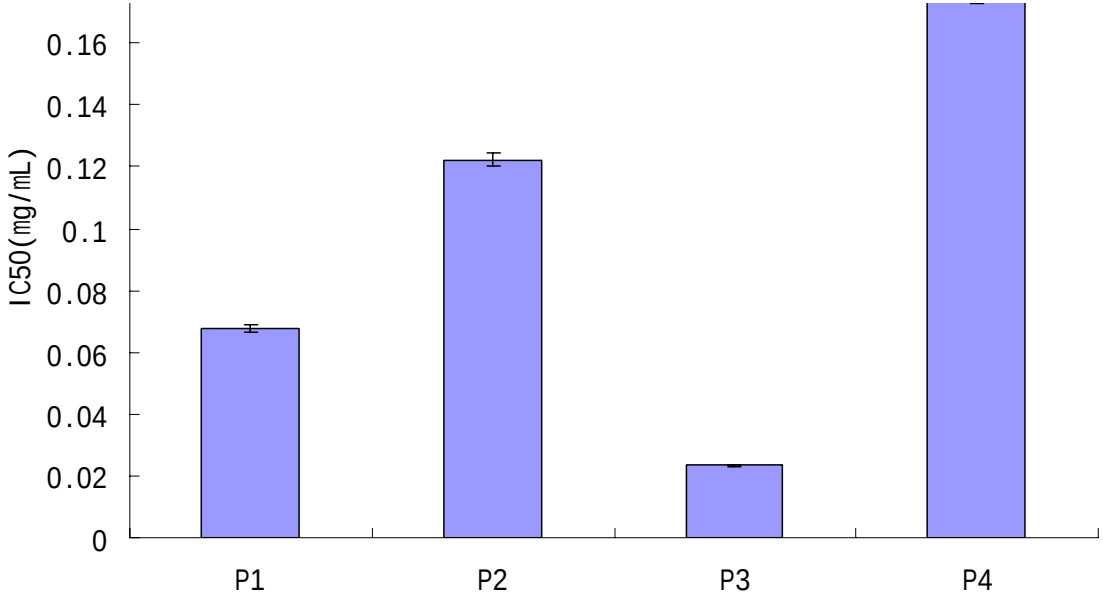
2.1 Separation and purification of angiotensin Ⅰ-converting enzyme inhibitor from walnut protein

- ACE inhibitor was purified step by step;
- Walnut protein was hydrolyzed by pepsin;
- Inhibitory activity of 1mg/mL hydrolysate was 20.79%;
- Among them, faction 0-5kDa was better than 5-10 kDa.

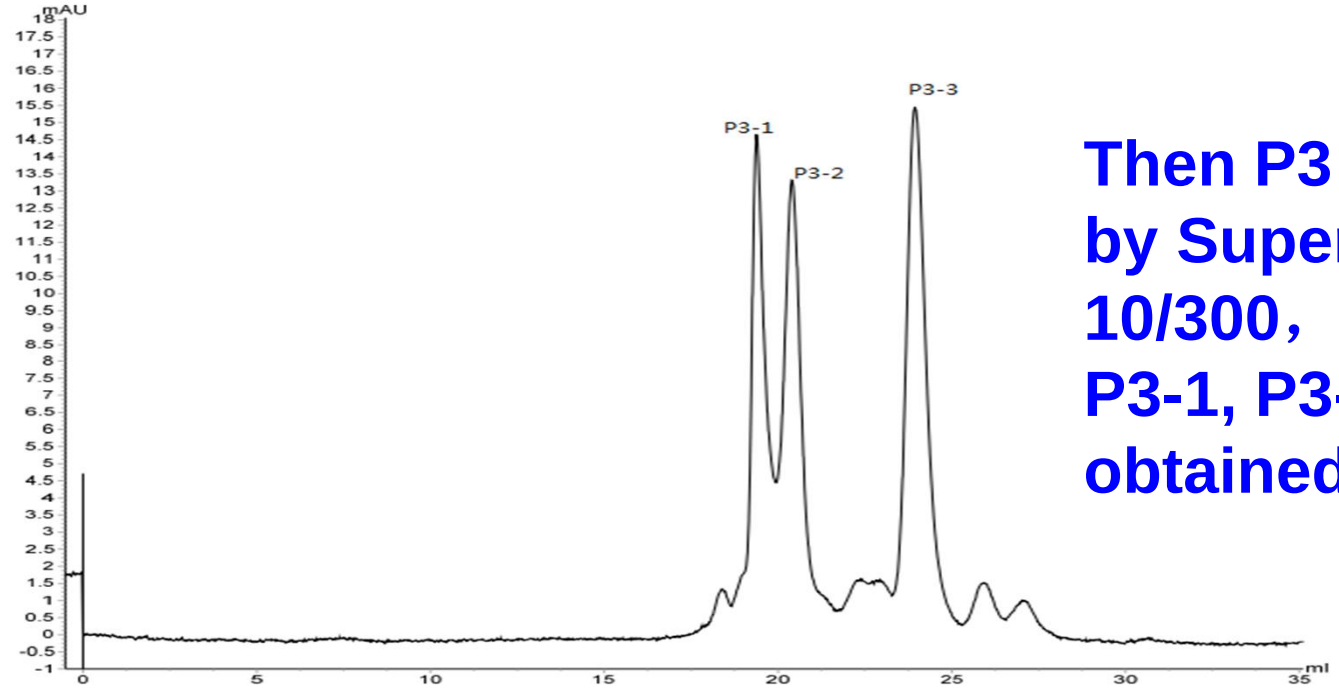
MW range (kDa)	0～5	5～10
IC_{50}(μg/mL)	40.00 ± 0.03	126.34 ± 0.02



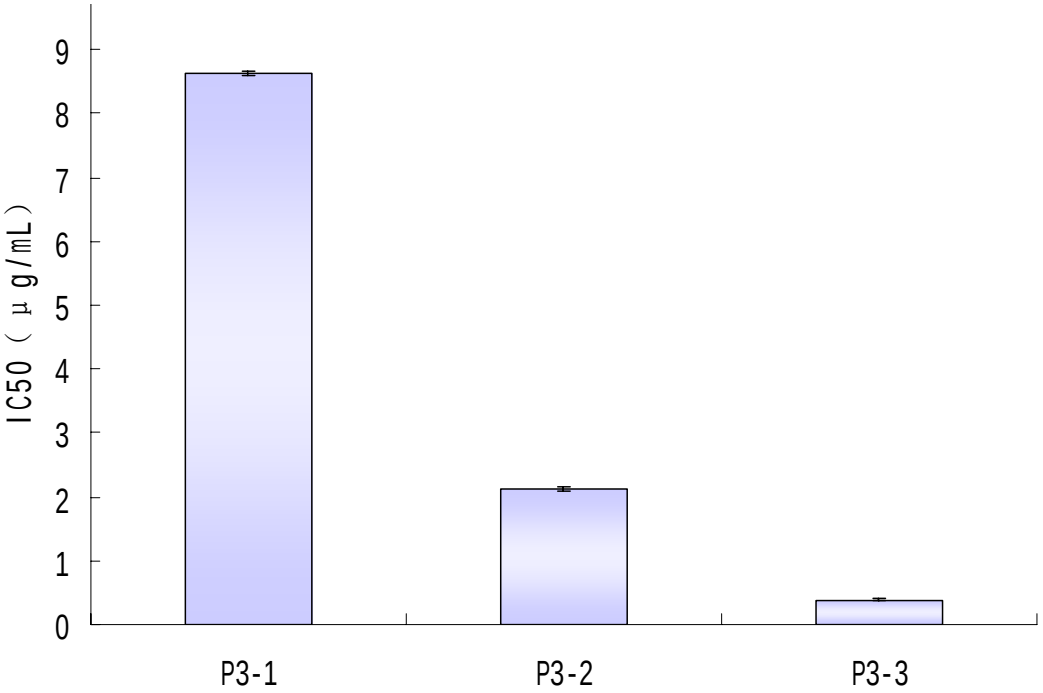
Fraction 0～5 kDa was Gel filtrated by Sephadex G-25, and four peaks P1,P2, P3, P4 obtained



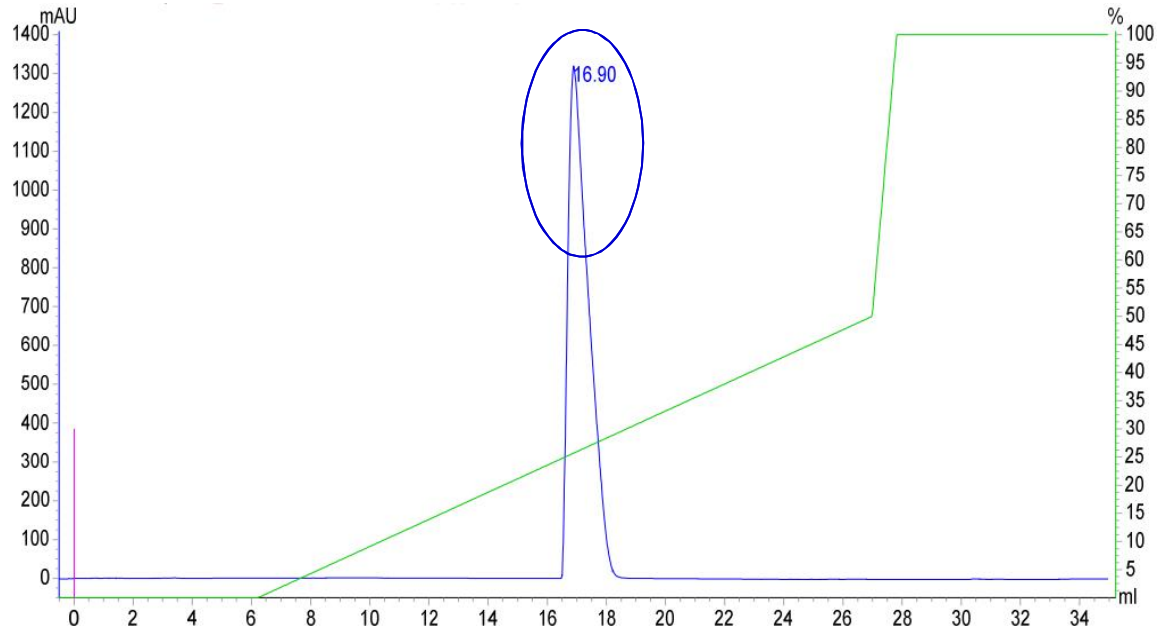
ACE inhibitory efficiency of P3 was the hightest and IC50=23.4μg/mL



Then P3 was seperated by Superdex™ peptide 10/300，three fractions P3-1, P3-2, P3-3 were obtained



P3-3 showed the hightest ACE inhibitory efficiency.



P3-3 was further purified by Reverse HPLC Zorbax SB-C18， the IC_{50} of ACE inhibitory efficiency was 0.32μg/mL

SEQUENCE LISTING

<110> 北京林业大学

<120> 高活性降血压肽及其制备方法

<130> PIDC120828

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Tyr Glu Pro
1

Structure of P3-3 was sequenced as：

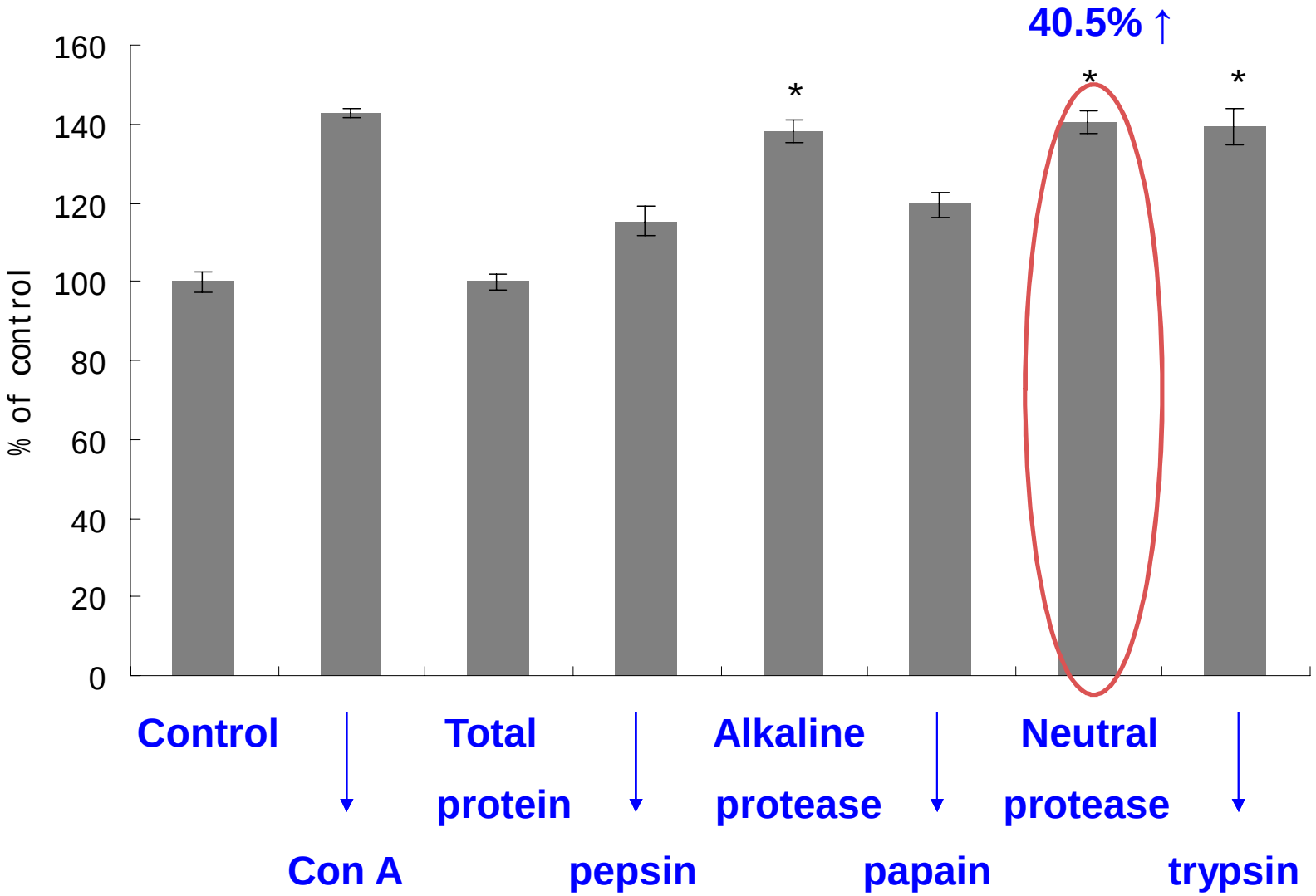
Tyr-Glu-Pro

Digestion Stability of ACE inhibitory peptide Tyr-Glu-Pro from walnut protein hydrolysate

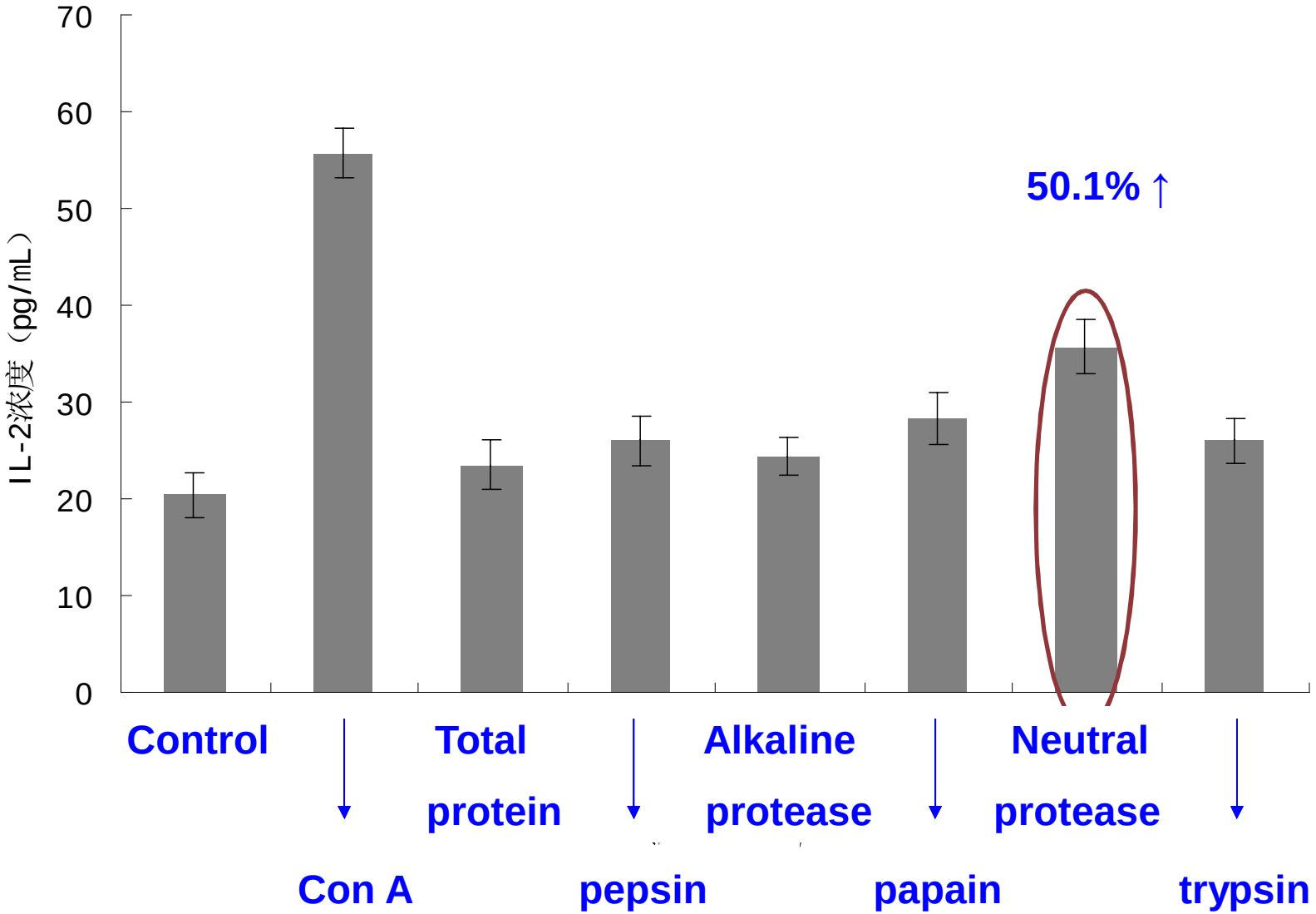
	Before digestion	After digestion
IC_{50}(μg/mL)	0.32 ± 0.01	0.36 ± 0.02

2.2 Promotion of spleen lymphocyte and macrophage functions induced by protease hydrolysates of walnut protein

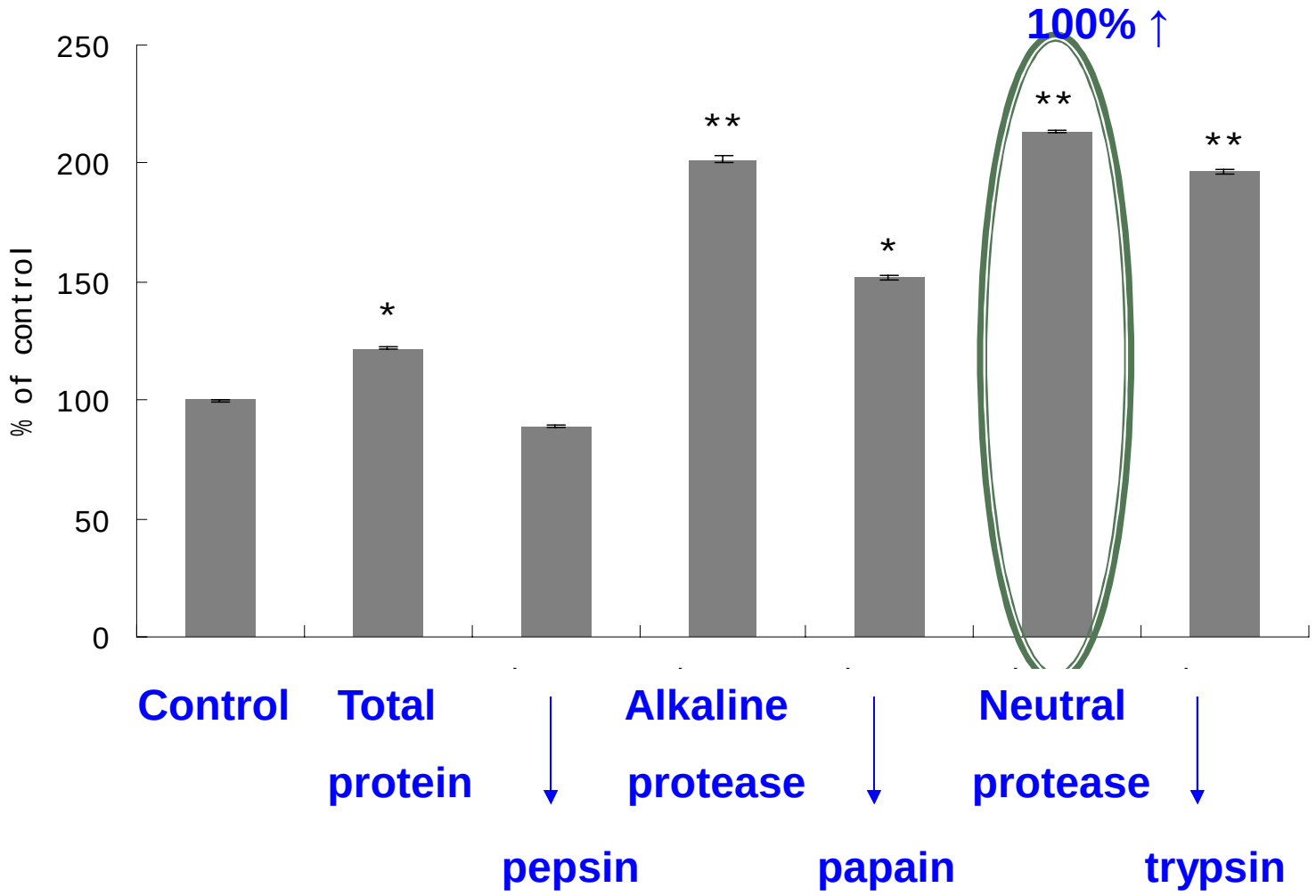
- Walnut protein hydrolysates were prepared with pepsin, trypsin, alkaline protease, papain and neutral protease respectively;
- Bioactivities of walnut protein and its hydrolysates on spleen lymphocyte and macrophage function.



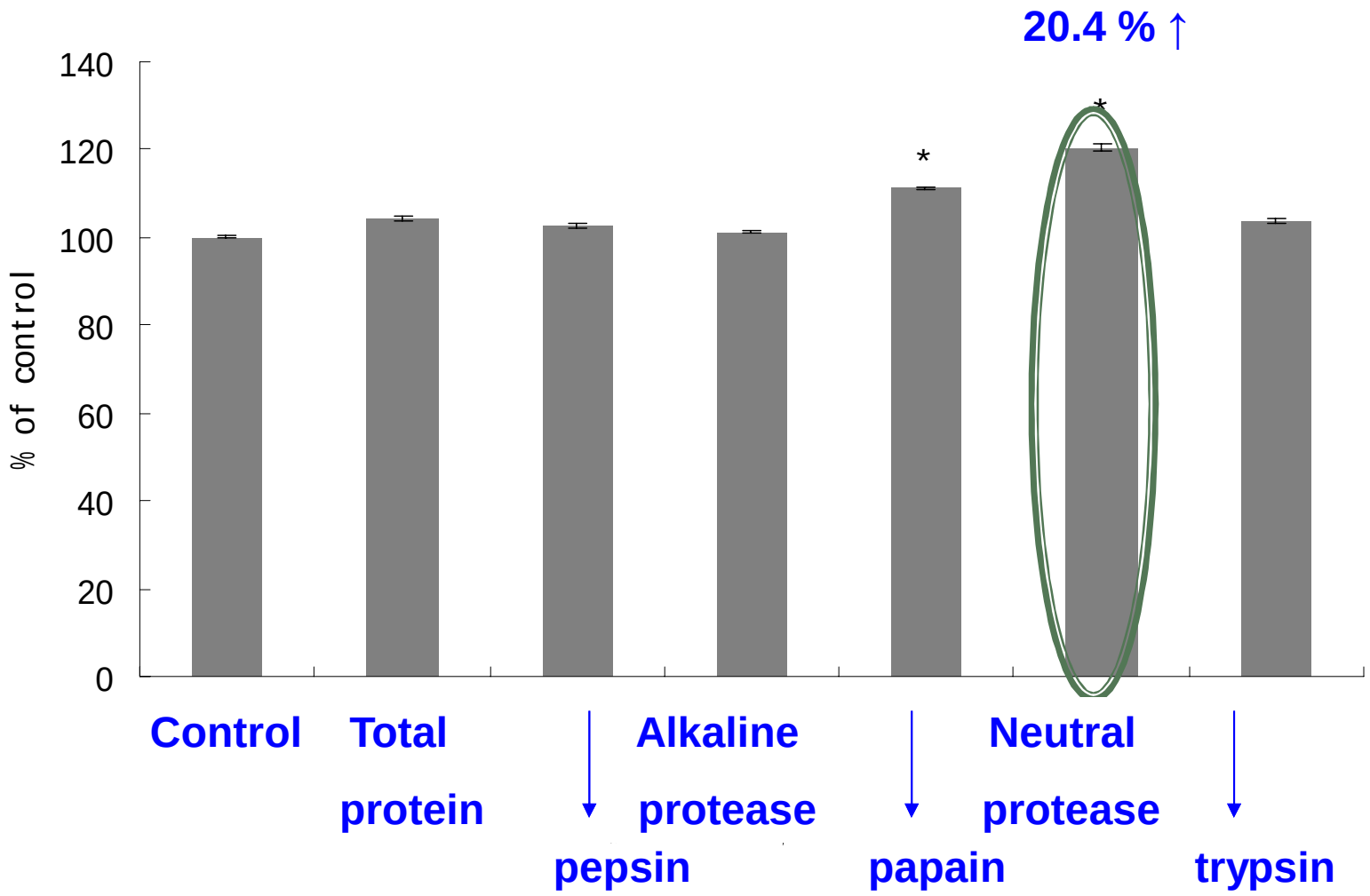
Effect of walnut protein and its hydrolysates (0.5 mg/mL) on spleen lymphocyte proliferation



Effect of walnut protein and its hydrolysates (0.5 mg/mL) on IL-2 secretion of spleen lymphocyte



Effect of walnut protein and its hydrolysates (0.5 mg/mL) on macrophage phagocytosis



Effect of walnut protein and its hydrolysates (0.5 mg/mL) on NO production by macrophage

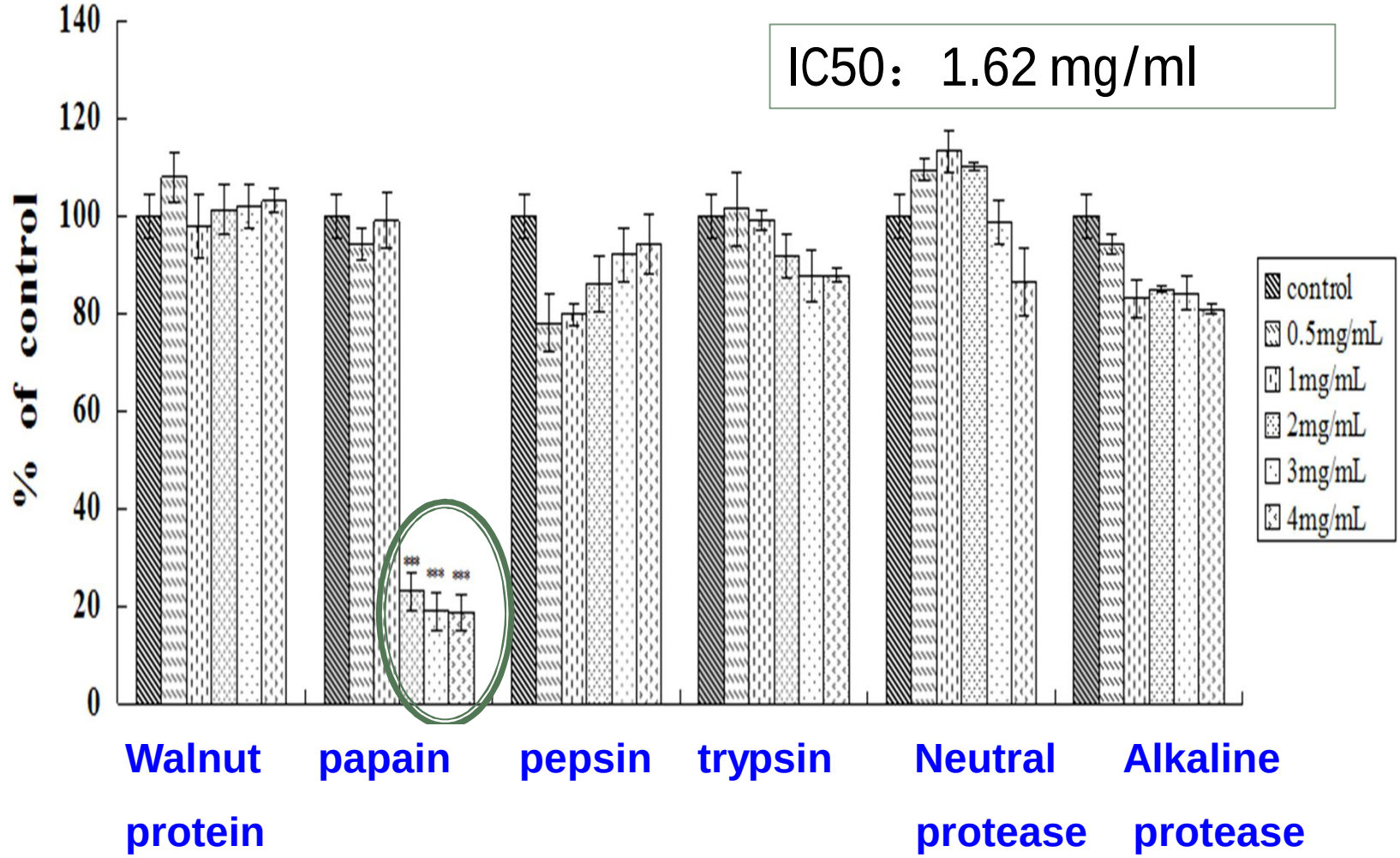
Conclusion:

- Walnut protein and its hydrolysates could promote the function of spleen lymphocyte and macrophage;
- The hydrolysates showed stronger effects than walnut protein, in which neutral protease was the strongest.

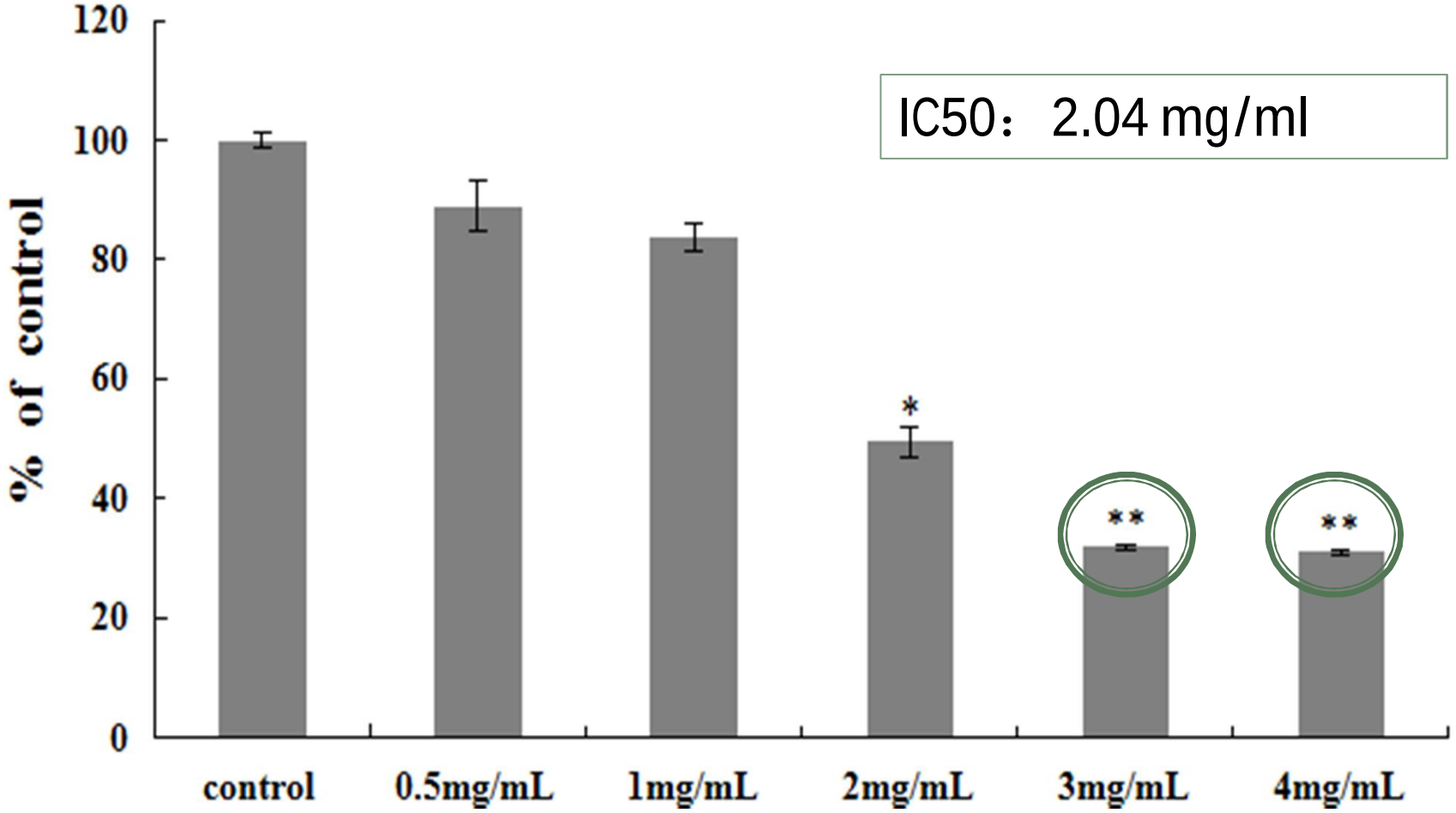
2.3 Inhibition of proliferation induced by hydrolysates of walnut protein on tumor cells

Three kinds of tumor cells were used：

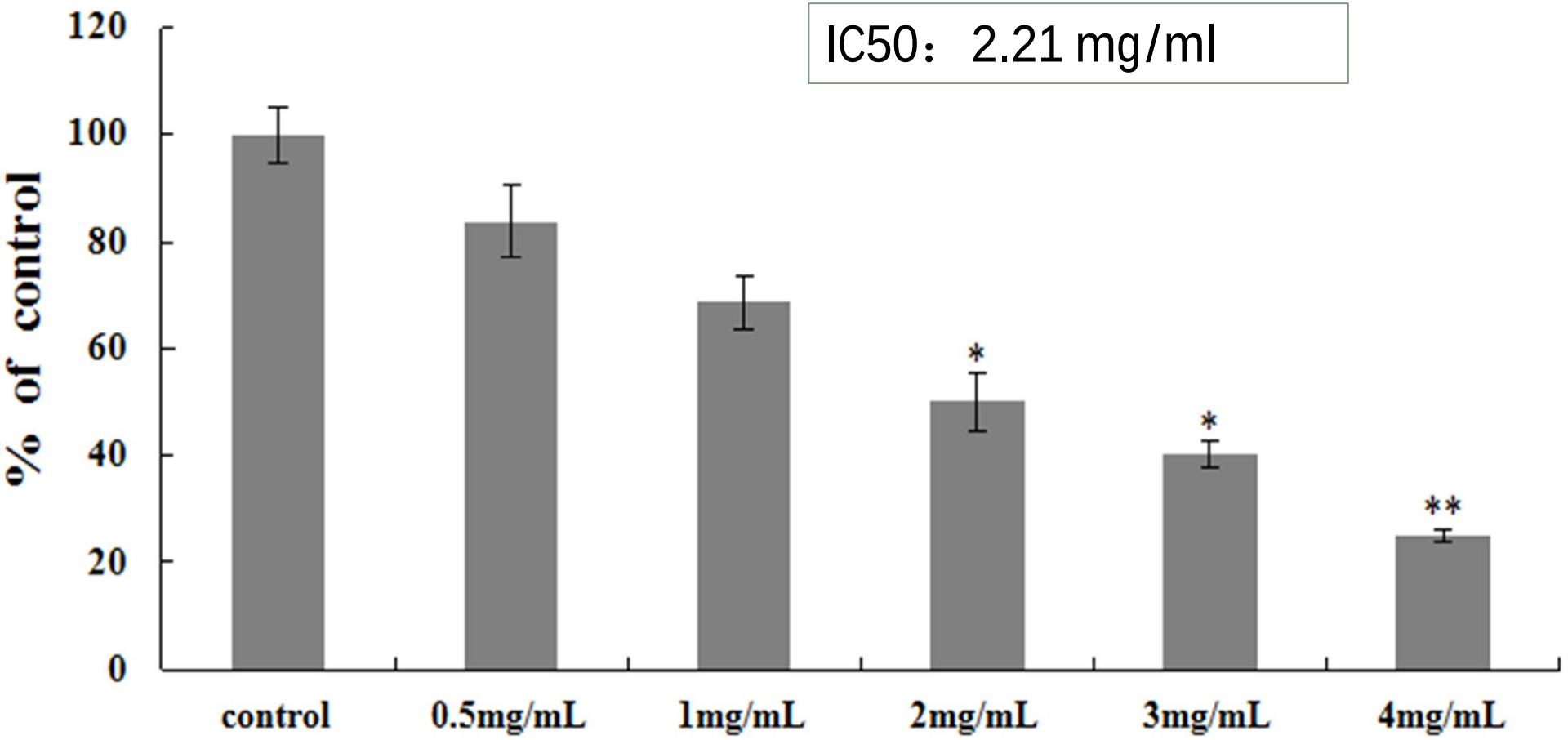
- **human breast cancer cells （MCF-7）**
- **human colon carcinoma cell （Caco-2）**
- **human cervical carcinoma cell （Hela）**
- **rat small intestinal crypt epithelial cell （ICE-6, not tumor cell）**



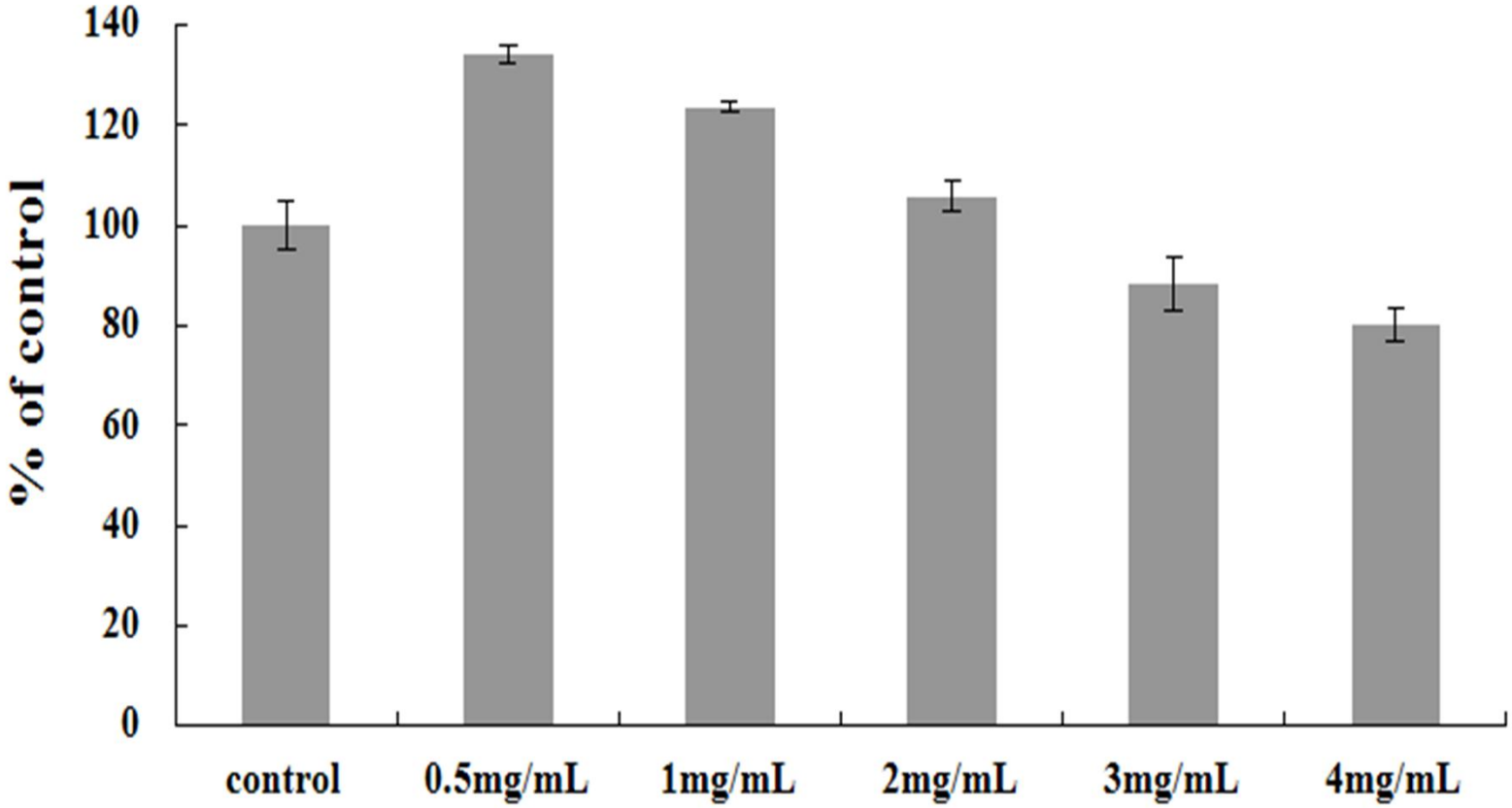
Inhibition of proliferation of MCF-7 induced by walnut protein and hydrolysates



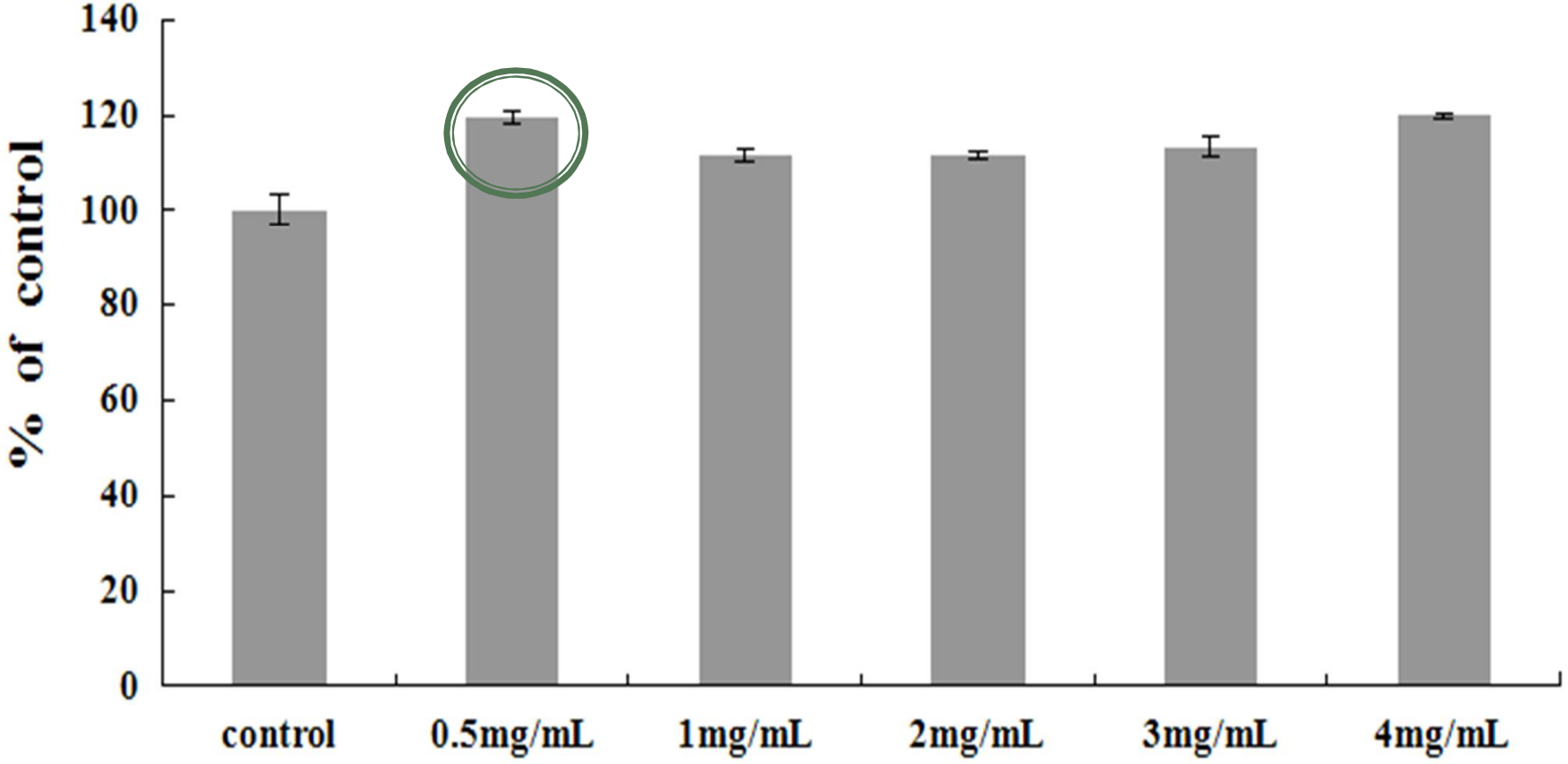
Inhibition of proliferation induced by papain hydrolysates of walnut protein on Caco-2



Inhibition of proliferation induced by papain hydrolysates of walnut protein on Hela



Inhibition of proliferation induced by papain hydrolysates of walnut protein on non-tumor cell IEC-6



Effect of walnut protein papain hydrolysates (0.5 mg/mL) on spleen lymphocyte proliferation

Conclusion：

- **Papain hydrolysates of walnut protein showed significant inhibition on the proliferation of tumor cell MCF-7, Caco-2 and Hela;**
- **Papain hydrolysates of walnut protein showed insignificant inhibition on the proliferation of non-tumor cell IEC-6, but could promote the proliferation of spleen lymphocyte.**

- **Research Team**

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